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**The Structure of the Alcohol and Anesthetic Binding Site  
in the Strychnine-Sensitive Glycine Receptor**

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**The Structure of the Alcohol and Anesthetic Binding Site  
in the Strychnine-Sensitive Glycine Receptor**

by

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# **The Structure of the Alcohol and Anesthetic Binding Site in the Strychnine-Sensitive Glycine Receptor**

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Ingrid Ann Lobo, Ph.D.

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The inhibitory glycine receptor is a target for both alcohols and volatile anesthetics. The function of strychnine-sensitive glycine receptors is enhanced in the presence of clinically relevant concentrations of ethanol and longer chain alcohols as well as volatile anesthetics. Site-directed mutagenesis techniques have identified residues in transmembrane segment (TM) one (I229), two (S267) and three (A288) that mediate the effects of alcohols and anesthetics, and drug binding is hypothesized to involve all amino acids from all four transmembrane segments. Here, by the use of crosslinking studies in receptors expressed in *Xenopus laevis* oocytes, we determined that S267 and A288 are near-neighbors that face one another in three-dimensional space. This provided an improved model of orientation for these two transmembrane segments and provides insight towards the location and role of the TM2-TM3 interface. Second, changes in the accessibility to the binding cavity and changes in the volume of this binding cavity were examined during channel gating for amino acids in all four TMs using mutagenesis and

sulfhydryl-specific reagents of different lengths. S267C was accessible to short chain (C3-C8) methanethiosulfonate (MTS) compounds in both open and closed states, but was only accessible to longer chain (C10-C16) MTS compounds in the open state. Reaction with S267C was faster in the open state. Mutated residues more intracellular than M263C in TM2 did not react, indicating a floor of the cavity. I229C and A288C showed state-dependent reaction with MTS only in the presence of agonist. Reaction of propyl MTS with A288C receptors abolished the effect of the anesthetic isoflurane, providing strong support that A288C is contributing to a binding site for alcohols and anesthetics. Four of twelve mutants tested in TM4 (W407C, I409C, Y410C and K411C) reacted with propyl MTS, providing information on which amino acids were in water-accessible positions and thus possible candidates for involvement in alcohol and anesthetic binding. These data demonstrate that the conformational changes accompanying channel gating increase accessibility to amino acids critical for drug action, which may provide a mechanism by which alcohols and anesthetics can act on glycine (and likely other) receptors.

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## Abbreviations

5-HT <sub>3</sub>	5-hydroxytryptamine type 3
cDNA	complementary deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EC	effective concentration
GABA	$\gamma$ -aminobutyric acid
GlyR	glycine receptor
nAChR	nicotinic acetylcholine receptor
SCAM	substituted cysteine accessibility method
LGIC	ligand-gated ion channel
MBS	modified Barth's solution
MTS	methanethiosulfonate
NMDA	N-methyl-D-aspartate acid
pCMBS <sup>-</sup>	<i>para</i> -chloromercuribenzene sulfonate
TM	transmembrane segment
WT	wild type

Amino acids positions are named using their standard single letter amino acid code and the numerical position of the amino acids in the primary amino acid sequence. For example a serine at position 267 is abbreviated S267.

Mutant amino acids positions are named by their standard single letter amino acid code from the wild type and numerical position of the amino acid in the primary protein sequence, followed by the one letter amino acid code of the introduced, mutant amino acid. For example a serine mutated to cysteine at position 267 is abbreviated S267C.

# **1.0 Introduction**

## **1.1 Alcohols and Anesthetics**

Alcohol has been used for at least 7000 years. The earliest known wine was discovered in archaeological excavations of a Neolithic village in present day Iran (McGovern et al., 1996). Examples of alcohol use abound in history from the Romans, who celebrated Dionysus, the god of wine, to social drinkers of the present day.

Ethanol can be described as an “organic derivative of water” where with a single ethyl group replaces one of the hydrogen atoms of a water molecule (McMurry, 1996). This seemingly small change results in the physical and the behavioral properties of alcohol. Alcohol is a central nervous system depressant, which means it depresses normal brain functions. Some of the intoxicating effects of alcohol include relaxation, sedation, motor incoordination, and impairments of cognition and memory. While many people enjoy pleasurable effects of alcohol, others abuse this drug. Alcoholism is a disease defined by an individual’s need for alcohol and includes craving for and a physical dependence on this drug. Understanding the molecular basis for this disease is key to effective treatment and prevention.

It is difficult to imagine most modern day medical operations without general anesthesia. Since 1846, when general anesthesia was first demonstrated, these drugs have been invaluable in operating rooms. Anesthetics cause immobility, sedation, hypnosis, and amnesia, and often include further components, such as relaxation,

analgesia, and anxiety reduction (Rudolph and Antkowiak, 2004). These drugs ensure that a patient does not move during surgery or remember the procedure. Some volatile (inhaled) anesthetics, such as chloroform, were used clinically in the past, and others, such as isoflurane, are still used today. Understanding how anesthetics act is important to areas of neuroscience involving consciousness, arousal, and perception. Additionally, understanding how anesthetics work at a molecular level could help devise administration of safer anesthetics with fewer, and less dangerous, side effects. Like alcohols, anesthetics are central nervous system depressants. Overall, alcohols and anesthetics share many behavioral and pharmacological effects.

Alcohols and volatile anesthetics are drugs that affect the brain, but the molecular mechanism by which these drugs cause their effects has been controversial. Initially, alcohols and anesthetics were believed to disrupt the membranes surrounding cells in a nonspecific manner. This view has changed in the past 20 years, as these drugs were shown to have direct effects on proteins in the brain. Now, alcohols and anesthetics are known to have specific protein targets in the brain, including ion channels.

## **1.2 Ligand-Gated Ion Channels**

Ion channels are transmembrane proteins, formed from one or more protein subunits. Shaped like tunnels, they form pores through the plasma membrane with gates that open and close to allow ions to diffuse down their chemical gradient and move in or out of a cell. Ions are unequally distributed, creating a separation of charge across a membrane, called an electrical potential. When an ion channel is open, a million ions can

flow in or out of the cell per second, resulting in an electrical signal or current (Siegelbaum and Koester, 2000).

There are two major classes of ion channels defined by the way the channel opens: voltage-gated and ligand-gated. Voltage-gated ion channels have sensors for the electrical potential across the membrane and open when the cell is at a specific membrane potential. Ligand-gated ion channels (LGIC) open when a specific chemical signal, or neurotransmitter, is released from a neuron, diffuses through a gap known as a synapse, and binds to receptors on ion channels of a second neuron. The binding of the neurotransmitter causes the gates of ion channels to open. In this way, the ligand-gated ion channel family of receptors mediate fast communication in the nervous system, allowing for orchestration of our physical and mental activities (Lobo and Harris, 2004).

Diseases called channelopathies occur when ion channels do not function properly. Some examples are epilepsy, cystic fibrosis, heart arrhythmia and high blood pressure. Ion channels are also the target of many types of drugs and toxins, which can alter the fundamental communication between cells. Of interest here are the changes caused by alcohols and anesthetics. Multiple ligand-gated ion channels are affected by anesthetics and alcohols, including the glycine and  $\gamma$ -aminobutyric acid (GABA) receptors as well as glutamate, acetylcholine, and serotonin receptors.

The pentameric ligand-gated ion channels are believed to have a common evolutionary origin (Ortells and Lunt, 1995). They have notable conservation in their sequence alignments (Le Novere and Changeux, 1999). Included in this family of

channels are the inhibitory glycine, GABA<sub>A</sub> and GABA<sub>C</sub> receptors, and the excitatory nicotinic acetylcholine and serotonin 5-HT<sub>3</sub> receptors.

### **1.3 Glycine Receptors**

The two major inhibitory neurotransmitters in the central nervous system are glycine and GABA. Both transmitters bind to their respective receptors to activate chloride-sensitive channel proteins positioned on the postsynaptic membrane and hyperpolarize neurons. This inhibits electrical activity in nerve cells.

Glycine is the simplest of all of the amino acids. In addition to its many metabolic roles, it was shown to have depressant actions on spinal neurons in 1960 by Curtis and Watkins (Rajendra et al., 1997), and discovered to be a neurotransmitter in 1965 by Aprison and Werman (Aprison, 1990). When glycine is released from presynaptic sites onto glycine receptors on the postsynaptic membrane, the ion channels open. This allows an influx of chloride and bicarbonate ions in adult neurons, hyperpolarizing the cell, and stabilizing the resting potential of the neuron.

The glycine receptor (GlyR) and GABA receptor are highly homologous; however, GlyRs predominate in the spinal cord and brain stem, while GABA receptors are more abundant in the cortex and cerebellum (Langosch, 1995; Legendre, 2001). Along with their importance in the spinal cord, glycine receptors have been identified in many locations in the brain as well, including the cortex (Breustedt et al., 2004), retina (Pourcho and Goebel, 1990), central auditory system (Wentholt and Hunter, 1990), ventrolateral medulla (involved with respiration) (Ezure et al., 2003), hippocampus



(Chattipakorn and McMahon, 2002; Chattipakorn and McMahon, 2003), amygdala (McCool and Farroni, 2001), and ventral tegmental area (involved in reward) (Ye et al., 1998a). Glycine receptor  $\beta$  subunits are found in many regions with no known detectable  $\alpha$  subunits (Betz, 1991; Legendre, 2001). Additionally, glycine receptor mRNAs have been localized in many other brain regions by *in situ* hybridization where functional glycinergic synapses have not yet been shown to exist (Legendre, 2001).

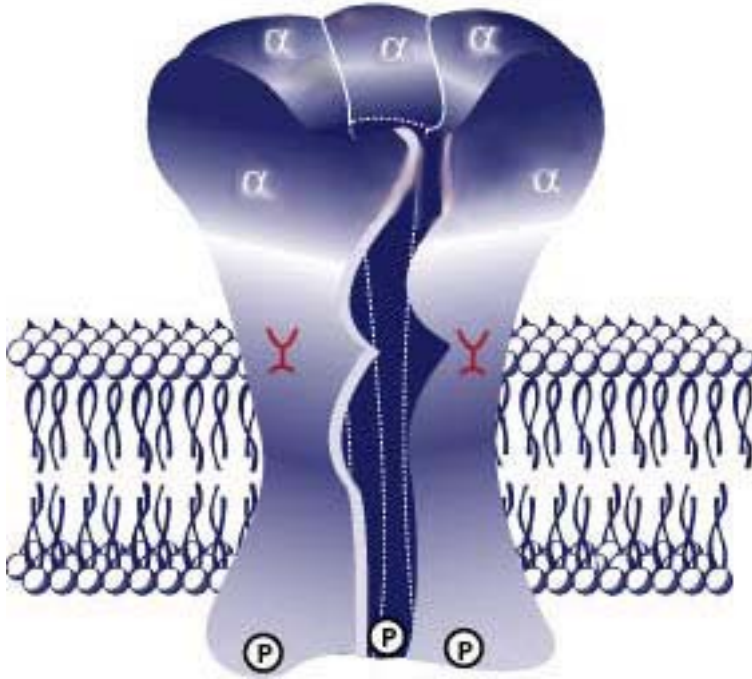
#### **1.4 Glycine Receptor Structure**

The glycine receptor is a membrane-bound protein composed of five subunits arranged around a central pore, with a stoichiometry of three  $\alpha$  subunits and two  $\beta$  subunits *in vivo* (Betz, 1991). Because of the glycine receptor's high affinity binding to its competitive antagonist strychnine, GlyRs were the first of the Cys-loop proteins to be isolated from the nervous system of mammals (Bechade et al., 1994; Pfeiffer et al., 1982). To date, four alpha subunits ( $\alpha 1$ - $\alpha 4$ ) and one  $\beta$  subunit have been identified (Grenningloh et al., 1990; Legendre, 2001; Rajendra et al., 1997).

The composition of GlyRs changes with development. Embryonic GlyRs in immature neurons are predominantly  $\alpha 2$  homomeric receptors, and the glycinergic synapses can be excitatory (Legendre, 2001; Takahashi et al., 1992; Tapia and Aguayo, 1998). In adults, glycine receptors are inhibitory and are most commonly composed of three  $\alpha 1$  (each approximately 48 kDa) and two  $\beta$  subunit (each approximately 58 kDa) to

form a 250 kDa size protein (Betz, 1991). This pentameric assembly was shown to be the most common composition in crosslinking studies (Langosch et al., 1988).

Overall homomeric GlyRs form functioning receptors with properties similar to those of native, adult receptors making them useful for studies involving electrophysiology and mutagenesis (Mascia et al., 1996a). When expressed in a heterologous system, such as the *Xenopus laevis* oocyte used in these studies, GlyR  $\alpha 1$  subunits can assemble homomERICALLY (Figure 1).

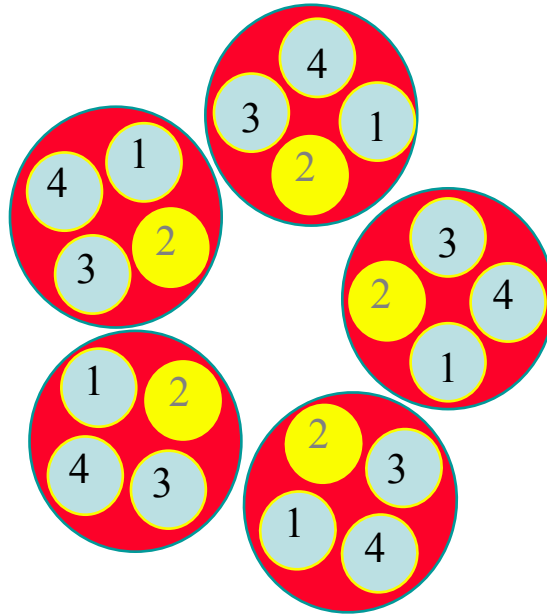


**Figure 1. Depiction of a glycine receptor.** Glycine receptors span the lipid bilayer and are composed of five subunits surrounding a central ion channel. In *Xenopus* oocytes, five, identical  $\alpha 1$  wild type or mutant subunits can be heterologously expressed and studied.

Each  $\alpha 1$  subunit is an integral membrane protein with four transmembrane segments (TM1-TM4). Hydropathy analysis of the glycine receptor sequence predicted that these four hydrophobic segments spanned the membrane and had alpha helical conformations (Cascio, 2004). Experiments involving mutagenesis of TM1-TM4 have supported this prediction. Additionally, the recent cryo-electron micrograph data on the *Torpedo* nAChR provided good evidence for an alpha helical topology for all four segments (Miyazawa et al., 2003). At present, the cryo-electron micrograph data is not high resolution, so questions remain over the exact positioning of the amino acids. Also, whether the *Torpedo* structure can be applied, without modification, to every member of the cys-loop family remains to be demonstrated. Because there is little sequence identity between nAChRs and inhibitory glycine and GABA receptors, even in the conserved membrane segments, it is possible that differences in structure exist within the receptor family.

The TM2 domains form the wall of the anionic pore and is believed to be alpha helical in secondary structure (Legendre, 2001). The alpha helical periodicity of TM2 is well characterized biochemically by use of probing with the substituted cysteine accessibility method (described in detail later). These studies have shown periodic accessibility of thiol-specific reagents in nAChRs (Akabas et al., 1994; Wilson and Karlin, 2001; Zhang and Karlin, 1998), GABA<sub>A</sub>Rs (Goren et al., 2004; Williams and Akabas, 1999; Xu and Akabas, 1996) and serotonin receptors (Panicker et al., 2002; Reeves et al., 2001). This alpha helix has a kink due to a bulky, conserved leucine, which lines a critical part of the pore and is postulated to function as a pore blocking site and be

involved with receptor gating (Chang and Weiss, 1998; Chang and Weiss, 1999; Shan et al., 2002; Unwin, 1995; Unwin, 1998). TM2 also contains other amino acids responsible for determining pore diameter and ion charge selectivity, and has rings of positively charged arginines and the top and bottom of the channel pore (Keramidas et al., 2000; Keramidas et al., 2002; Keramidas et al., 2004; Lee et al., 2003). Ion charge selectivity is defined by the most intracellular section of TM2 of the glycine receptor, which also corresponds with the narrowest region of the ion channel pore (Keramidas et al., 2000; Keramidas et al., 2002; Keramidas et al., 2004; Lee et al., 2003). An illustration of the five GlyR subunits and the four transmembrane segments of each subunit is shown in Figure 2.

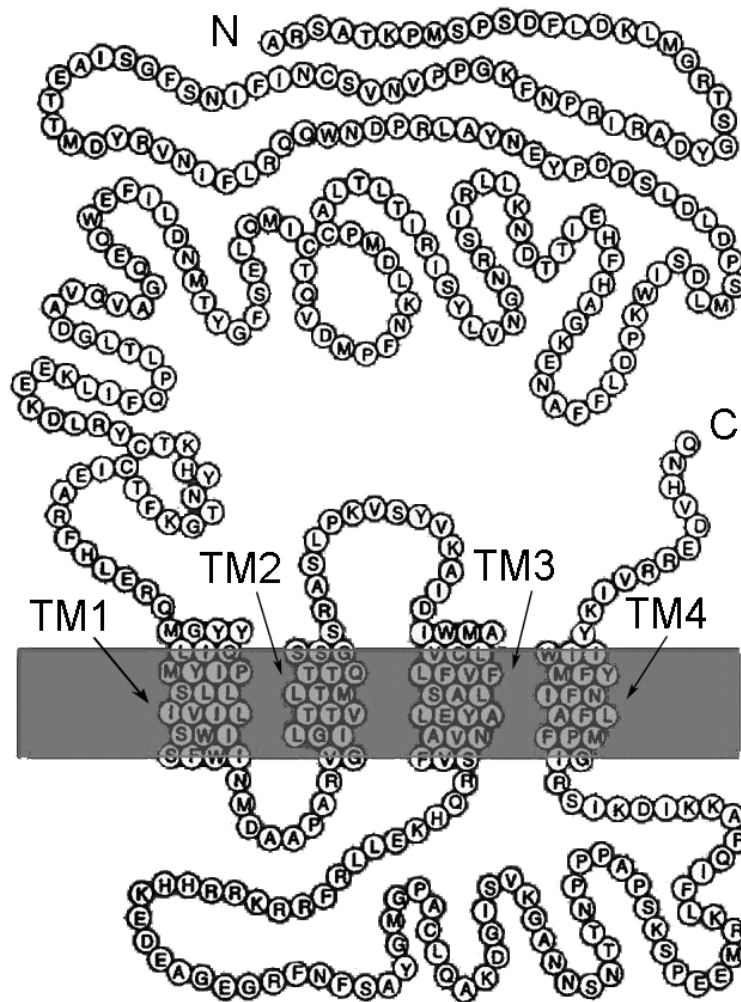


**Figure 2: A schematic view of a glycine receptor membrane-spanning domain.** Viewed from the extracellular surface of the cell, the receptor is composed of five subunits. Each receptor subunit is believed to be a four-helical bundle in a clockwise in arrangement (the transmembrane segments are labeled 1-4). The second transmembrane segment of each subunit lines the channel pore.

As with other ion channels, GlyRs are not present in high quantities in mammals, *in vivo*. While, nicotinic acetylcholine receptors can be isolated in enriched quantities from the electric organ of *Torpedo* electric fish (Unwin, 1998), there is no known natural, enriched source to isolate glycine receptor protein from for structural analysis (Cascio, 2004). Because of this, nAChRs have been better characterized than the other members of its subtype in the ligand-gated ion channel family. For this reason, this family of receptors is sometimes referred to as the nicotinicoid receptors. Recent work has progressed with overexpressing  $\alpha 1$  glycine receptor subunits in insect cells infected with baculovirus (Cascio et al., 1993; Cascio et al., 2001; Morr et al., 1995). As yet, a high-resolution crystal structure of the protein is not available.

The fact that we do not have high-resolution crystal structure data for the glycine receptor, or any other member of the ligand-gated ion channel family, leaves structural analysis in the realm of mutagenesis, biochemical manipulations and computer modeling. This has provided insight into details of the glycine receptor structure, and hopefully much of this data will be confirmed when a glycine receptor crystal structure is imaged.

Each glycine receptor subunit has a large, extracellular, N-terminal region, four transmembrane segments, a large intracellular linker between TM3 and TM4, and a short, extracellular, C-terminal tail (Langosch, 1995) (Figure 3).



**Figure 3. Schematic of a single glycine receptor  $\alpha$  subunit.** Each glycine receptor subunit has a large, extracellular, N-terminal region, including the glycine binding site and two cys-loops (disulfide bound loops). The protein chain crosses the membrane four times to give it four transmembrane segments. There is a large intracellular linker loop between transmembrane segments three and four, and the C-terminal end of the protein is extracellular.



The N-terminal domain of the protein forms the ligand (glycine) binding domain. There are two highly conserved disulfide bonded loops which are important for the receptor function and which are involved with ligand binding (Rajendra et al., 1995). Because of the conservation of the cys-loops in these channels, this family is sometimes called “cys-loop” ion channels. Uncoupling the first and second cysteine loop abolishes glycine receptor current, indicating that these loops play an important role in receptor stability and assembly (Rajendra et al., 1997). Recently, the 2.7 Å structure of the acetylcholine binding protein was elucidated from crystallizing the protein from a freshwater snail (*Lymnaea stagnalis*) DNA library (Brejc et al., 2001). This protein is a homopentamer, and each subunit is homologous to the N-terminal extracellular halves of the subunits of the ionotropic receptors (Brejc et al., 2001), thus providing structural details on the N-terminal domain. Also, glycine’s competitive antagonist strychnine has a binding site near that of glycine (Vandenberg et al., 1992a; Vandenberg et al., 1992b).

Much recent effort has focused attention on how the signal from ligand binding is transduced to the transmembrane regions and causes the pore to open. Studies have found that the ligand binding domain has loops which interact with the linker region between TM2 and TM3 to couple agonist binding to the transmembrane domain (Kash et al., 2003). The TM2-TM3 linker has also been shown to have increased accessibility as a result of channel gating (Lynch et al., 2001). Still, the signal transduction mechanism from the point of glycine binding in the N-terminal region to the point of pore opening is not entirely understood.

Low expression of glycine receptors or receptors with reduced activity due to specific mutations cause channelopathies in humans, such as hyperekplexia (human startle disease) (Breitinger and Becker, 2002; Legendre, 2001). Mutations resulting in startle disease have been identified in the TM1-TM2 intracellular loop, in TM2 and in the extracellular TM2-TM3 linker loop (Legendre, 2001; Rajendra et al., 1997; Schofield, 2001).

The large intracellular linker loop between TM3 and TM4 is the most diverse in terms of sequence between the LGICs. The region is responsible for protein-protein contacts hypothesized to affect GlyR assembly, trafficking, clustering, targeting, turnover and modulation (Cascio, 2004). It contains phosphorylation sites as well as a binding site for gephyrin. Gephyrin is a cytoplasmic anchoring protein which was originally purified in the first isolation of the glycine receptor (Pfeiffer et al., 1982).

Of interest here is the transmembrane domain of the GlyR  $\alpha 1$  subunit, particularly the amino acids facing towards the center of each transmembrane helical bundle. This is the region of the protein where alcohols and anesthetics are believed to bind and cause their actions.

## **1.5 Drug Effects**

Glycine receptors are affected and modulated by a number of drugs including: alcohols (Aguayo and Pancetti, 1994; Celentano et al., 1988; Engblom and Akerman, 1991; Engblom et al., 1996; Mascia et al., 1996a; Mascia et al., 1996b; Mihic, 1999; Ye et al., 2001a; Ye et al., 2001b), volatile and intravenous anesthetics (Yamakura et al.,

2001) and inhalants (Beckstead et al., 2001; Beckstead et al., 2000). Here, we are concerned with the effects of alcohols and volatile anesthetics.

Clinically relevant concentrations of ethanol have been reported to potentiate the glycine receptor response in chick spinal cord neurons (Celentano et al., 1988), rat brain synaptoneurosomes (Engblom and Akerman, 1991), and cultured mouse hippocampal, cortical and spinal neurons (Aguayo and Pancetti, 1994; Aguayo et al., 1996).

Experiments performed on developing rat hypoglossal motor neurons showed that glycine receptors composed of  $\alpha 2$  subunits were less sensitive to ethanol than receptors composed of  $\alpha 1$  subunits (Eggers et al., 2000). Experiments in the ventral tegmental area neurons of rats have shown differential results from cell to cell. In 35% of cells, the glycine receptors were potentiated by ethanol (Ye et al., 2001a), and while in another 45% of glycine receptors were inhibited by ethanol (Ye et al., 2001b).

In heterologous expression systems (*Xenopus laevis* oocytes and HEK cells) the function of glycine receptors is also enhanced in the presence of clinically relevant concentrations of ethanol and longer chain alcohols (Krasowski and Harrison, 2000; Mascia et al., 1996a; Mascia et al., 1996b). Since glycine receptors are mediators of inhibition in the spinal cord and in some areas of the brain, they are likely involved in the sedative and anesthetic effects of alcohol. This hypothesis is supported by a study showing decreased alcohol effects in transgenic mice expressing a mutant, alcohol resistant,  $\alpha 1$  subunit (Findlay et al., 2002).

In the same way, volatile anesthetics have been shown to potentiate glycine receptors. Experiments have shown that volatile anesthetics enhance glycine-activated

chloride currents of glycine receptors in rat medullary neurons (Downie et al., 1996), dissociated rat hippocampal neurons (Kira et al., 1998), as well as in recombinant systems with transiently transfected cells (Harrison et al., 1993) and in *Xenopus* oocytes (Downie et al., 1996). Analysis of current data showed that the glycine receptor is one of the most credible candidates for mediating immobility caused by volatile anesthetics (Sonner et al., 2003). Both alcohols and volatile anesthetics enhance glycine receptors in a concentration dependent manner and shift the glycine concentration response curve to the left, without altering the maximal glycine response (Legendre, 2001).

GABA  $\rho 1$  receptors share sequence homology with GlyRs, and like GlyRs, they may be expressed homomerically. There exists a noteworthy difference between these two receptors: homomeric GABA  $\rho 1$  receptors are inhibited by ethanol, while homomeric GlyRs are enhanced by ethanol. Taking advantage of this difference, chimeric receptors were created using the sequences of both, leading the way to identifying the site of alcohol action on the glycine receptors (Mihic et al., 1997). Stemming from these studies, two amino acids were determined to be critical for alcohol and anesthetic action on the glycine receptor (as well as the homologous residues in the GABA<sub>A</sub> receptor), a site in TM2 (S267), as well as a residue in TM3 (A288) (Mihic et al., 1997). Mutations in homologous positions in different GABA<sub>A</sub> receptor subunits reduced ethanol potentiation when the mutations were in either the  $\alpha 2$  or the  $\beta 1$  subunits, but the same mutations in the  $\gamma 2L$  subunit had no effect (Ueno et al., 1999).

Mutagenesis at S267 showed that ethanol was only able to potentiate the glycine receptor if serine was replaced with a small amino acid, while substitution of larger

amino acids (such as valine) resulted in no effect, and substitution of even larger amino acids (such as tyrosine) resulted in inhibition (Ye et al., 1998b). Mutagenesis of A288 showed that the molecular volume of this position is negatively correlated with potentiation by volatile anesthetics (Yamakura et al., 1999). Exchange of the amino acids at these sites in TM2 and TM3 between the homomeric GABA  $\rho 1$  and Gly  $\alpha 1$  receptors switched the alcohol cutoffs in both receptors, suggesting that these residues controlled the size of an alcohol binding cavity (Wick et al., 1998). Also, coupling mutagenesis of the homologous critical positions (S270 and A291, along with the TM1 site L232) in the GABA<sub>A</sub> receptor and using volatile anesthetics of different sizes allowed for an estimate of the size of the cavity (Jenkins et al., 2001). These pieces of evidence suggest that amino acids in TM1, TM2 and TM3 are a part of a single alcohol and volatile anesthetic binding cavity.

Experiments on a constitutively active mutant receptor showed that ethanol and volatile anesthetics affect channel opening independent from ligand binding (Beckstead et al., 2002). A recent study that used increased atmospheric pressure as an antagonist of ethanol's actions suggested that there were multiple sites of ethanol action on the glycine receptor (Davies et al., 2004). While one site seems to be located in the most extracellular section of the transmembrane region, a second site may exist in the amino-terminus (A52) (Davies et al., 2004; Mascia et al., 1996b). Here, the focus was on the better studied drug binding site in the transmembrane region of each glycine receptor subunit.

## 1.6 SCAM and the MTS Reaction

Methanethiosulfonate (MTS) reagents and other thiol-specific compounds have been used for various purposes. Some are used as blocking, affinity labeling and reporter groups. Others may also be used as crosslinkers and as chemical modifiers of peptides and proteins (Dime, 1997). There are many differently sized, shaped and charged thiol-specific reagents available. Those used in the present studies included neutral alkyl MTS reagents of various carbon chain lengths, ranging from methyl MTS to hexadecyl MTS, as well as benzyl MTS and pCMBS<sup>-</sup>.

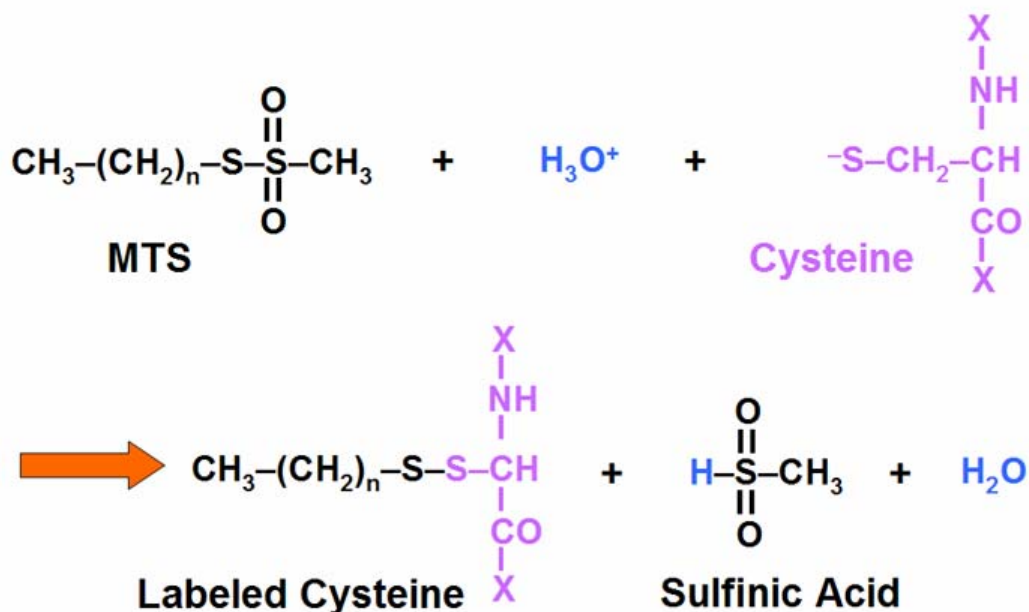
The substituted cysteine accessibility method (SCAM) couples site-directed mutagenesis and biochemical probing with MTS compounds. SCAM uses MTS reagents to explore the local environment of specific positions in receptors and to reveal environmental changes under different conditions (Karlin and Akabas, 1998). For example, SCAM allows changes in specific positions to be examined in the presence and absence of agonist or drug molecules. In electrophysiological experiments, reaction is measured by a change in the properties of the receptor after reaction, as a change in current.

Reaction with a cysteine occurs or does not occur, which can indicate distinct changes in local environment and accessibility to a specific position. Reaction also may occur at different rates. In these cases, reaction occurs more quickly at one position than another. The comparison of the reaction rates between different substituted cysteines can provide information about the local environment of a position in a protein. In particular, reaction depends upon two factors: accessibility and reactivity. Accessibility depends on

the access pathway from the bath solution to the cysteine and the steric and electrostatic factors encountered by the MTS molecule (Bali and Akabas, 2004; Karlin and Akabas, 1998). Reactivity depends upon the local environment surrounding the substituted cysteine, including the ionization state, the local electrostatic potential, the amount of time spent in the water-accessible surface and local steric changes at the cysteine (Karlin and Akabas, 1998).

If the MTS molecule is able to reach the substituted cysteine, then reaction of MTS requires that the sulfhydryl side chain of the cysteine is ionized and reactive. Ionization occurs predominantly in the presence of water. In the ionized state, the cysteine and MTS molecule can covalently react to label the cysteine, and reaction is at least  $5 \times 10^9$  faster with an ionized  $\text{S}^-$  than with an un-ionized  $\text{SH}$  (Karlin and Akabas, 1998). Meanwhile, on the lipid-accessible surface and in the protein interior, the dielectric constant for the environment is low, and ionization and MTS reaction are rare (Karlin and Akabas, 1998). Also produced in the reaction are sulfinic acid (which decomposes rapidly to low molecular weight, volatile products that do not affect receptor function) and water (Dime, 1997). MTS reaction with a cysteine indicates that the cysteine at a particular position is water-accessible (Figure 4).

# MTS Reaction Scheme



**Figure 4. Methanethiosulfonate (MTS) reaction scheme.** MTS reagents are sulfhydryl-specific compounds. In the presence of water, the amino acid cysteine is often in the ionized state with water removing a hydrogen atom from the cysteine side chain. In the ionized state, the cysteine and MTS molecule can covalently react to label the cysteine. Also produced in the reaction are sulfinic acid and water. MTS reaction with a cysteine indicates that the cysteine at a particular position is water-accessible.



The key question of whether S267C was important to the action of alcohols and anesthetics and part of a binding pocket was addressed by Mascia et al. (2000). Following mutation of a target GlyR amino acid residues to cysteine (S267C), an alkane thiol anesthetic or varieties of methanethiosulfonate compounds were used to covalently label this binding site (Mascia et al., 2000). By creating a method to covalently bind a drug molecule to the putative binding site, the receptor function was irreversibly enhanced. Also, the usual ability of octanol and isoflurane to enhance the receptor function was lost, indicating the action of alcohols and anesthetics stems from binding at a single binding pocket (Mascia et al., 2000).

The following dissertation branches from this work. Particularly, it aims to answer the questions of which other amino residues are involved in the binding pocket, how they are oriented with respect to one another and the relationship between binding and accessibility and receptor function.

## **1.7 Dissertation Aims**

**Overall Hypothesis and Goal:** The overall experimental plan is to structurally characterize the alcohol and volatile anesthetic binding site in the human glycine receptor. Particularly, the experiments will provide information concerning which residues contribute to the binding site, how the volume of the site changes with receptor gating, and the distance separating specific residues within the binding site itself. These results will contribute to an updated molecular model of the alcohol/anesthetic binding site and understanding of the mechanism by which drugs bind to it and affect it.

This plan includes three main aims:

**Aim 1:** To determine whether TM2 residue S267 and TM3 residue A288 face one another in three-dimensional space. Is an alcohol and volatile anesthetic binding pocket formed by residues in TM2 and TM3?

**Aim 2:** To determine whether the volume of this binding pocket differs when the channel is open in contrast with the size when the channel is closed. Do drugs stabilize the open state of the glycine receptor because of the volume they occupy in the drug binding cavity?

**Aim 3:** To determine whether amino acid residues in transmembrane segments 1 and 4 contribute to the alcohol and volatile anesthetic binding pocket.

## 1.8 Chapter Overview

Chapter 2 contains the experimental methods used to complete the aims studied. These include mutagenesis, *Xenopus* oocyte expression and two-electrode voltage clamp electrophysiology. It also describes the crosslinking and substituted cysteine accessibility experiments in full and the statistical analyses used.

Chapter 3 answers the questions posed in Aim 1 of my proposal. Intrасubunit contact points between the four transmembrane segments of ligand-gated ion channel subunits have not been defined experimentally. We tested whether two amino acids in TM2 (S267) and TM3 (A288), known to be critical for alcohol and volatile anesthetic

action, could crosslink by mutating both to cysteines and expressing the receptors in *Xenopus laevis* oocytes. In contrast with the wild type receptor and single cysteine mutants, the S267C/A288C double mutant displayed unusual responses including a tonic leak activity that was closed by strychnine and a run-down of the response upon repeated applications of glycine. We hypothesized that these characteristics were due to crosslinking of the two cysteines on opposing faces of these adjacent, alpha helical TMs. This would alter the movement of these two regions required for normal gating. To test this hypothesis, we used dithiothreitol to reduce the putative S267C-A288C disulfide bond. Reduction abolished the leak current and provided normal responses to glycine. Subsequent application of the crosslinking agent mercuric chloride caused the initial characteristics to return. Our evidence of disulfide formation between these two introduced cysteines defines the vertical position of TM3 with respect segment TM2, and shows that they face each other. This provides an improved model of orientation of these two TM segment and gives us some insight towards the location and role of the TM2-TM3 interface. Constraining this dynamic region of the glycine receptor with a disulfide bond, results in channels that cannot function normally. To our knowledge, this was the first demonstration of intrasubunit cross-linking of transmembrane segments in a ligand-gated ion channel.

Chapter 4 focuses on a question posed in Aim 3 of my proposal. A single amino acid (I229) in TM1, evidenced to be of interest in anesthetic action, was studied. This amino acid was mutated to cysteine and probed for reaction with sulfhydryl-specific reagents. I229C showed state-dependent reaction with MTS only in the presence of

agonist, indicating that the position is located in a water-filled environment. The effects of alcohols and anesthetics were determined for this mutant, and the ability of MTS reagents to interfere with drug binding was also examined.

Chapters 5 and 6 focus on Aim 2 of my dissertation proposal. The glycine receptor is a target for both alcohols and anesthetics, and certain amino acids in the  $\alpha 1$  subunit transmembrane segments are critical for drug effects. Introducing larger amino acids at these positions increases the potency of glycine, suggesting that introducing larger residues, or drug molecules, into the drug-binding cavity facilitates channel opening. A possible mechanism for these actions is that the volume of the cavity expands and contracts during channel opening and closing. To investigate this hypothesis, mutations for amino acids in TM2 (G256C, T259C, V260C, M263C, T264C, S267C, S270C) and TM3 (A288C) were individually expressed in *Xenopus laevis* oocytes. The ability of sulfhydryl-specific alkyl methanethiosulfonate (MTS) compounds of different lengths to covalently react with introduced cysteines in both the closed and open states of the receptor was determined. S267C was accessible to short chain (C3-C8) MTS in both open and closed states, but was only accessible to longer chain (C10-C16) MTS compounds in the open state. Reaction with S267C was faster in the open state. A288C showed state-dependent reaction with MTS, and reacted only in the presence of agonist. Additionally, reaction of A288C with propyl MTS blocked further potentiation by isoflurane, providing strong support that A288C is contributing to a binding site for alcohols and anesthetics. M263C and S270C were also accessible to MTS labeling. Mutated residues more intracellular than M263C did not react, indicating a floor of the

cavity. These data demonstrate that the conformational changes accompanying channel gating increase accessibility to amino acids critical for drug action in TM2 and TM3, which may provide a mechanism by which alcohols and anesthetics can act on glycine (and likely other) receptors.

Chapter 7 focuses on a question posed in Aim 3 of my proposal. A cysteine scan was completed for twelve amino acids of the TM4 domain. Each amino acid in this region was mutagenized to cysteine and tested for water-accessibility by probing these positions with sulfhydryl-specific reagents. Four mutants (W407C, I409C, Y410C and K411C) showed water-accessibility and irreversible changes in receptor function following reaction with propyl MTS. Since these native amino acids may be located in a water-filled environment, they may also be participants in the alcohol/anesthetic binding pocket. The effects of alcohols and anesthetics were determined for these mutants, and the ability of MTS reagents to interfere with drug binding was also examined.

Chapter 8 includes an overall discussion of these data and the conclusions that may be derived from these experiments.

## **2.0 Materials and Methods**

### **2.1 cDNA Preparation and Site-Directed Mutagenesis**

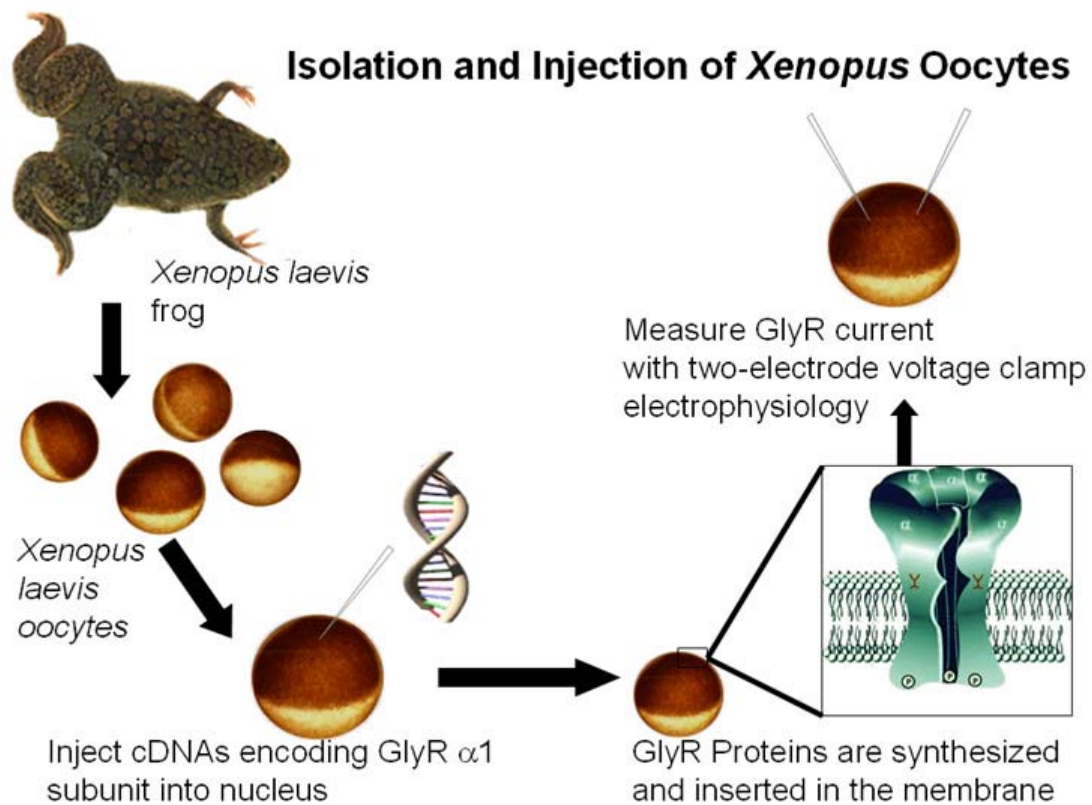
These studies used cDNAs encoding the wild type, human  $\alpha_1$  glycine receptor previously subcloned in the pBK-CMV N/B-200 vector. In order to introduce missense mutations into the receptor sequence, site-directed mutagenesis will be carried out using the Stratagene QuikChange<sup>TM</sup> Site-Directed Mutagenesis Kit (LaJolla, CA). Mutagenesis was also carried out on GlyR  $\alpha_1$ (S267C) cDNA subcloned previously in the laboratory in the pCIS2 vector. Mutagenic sense and antisense primers were designed for each desired mutation and ordered from Integrated DNA Technologies, Inc. (Coralville, IA). Partial sequencing of the mutated regions was performed by The University of Texas at Austin's Core Sequencing Facility to verify all introduced mutations. cDNA was purified and prepared using a QIAGEN mini- or maxi-prep kit (Valencia, CA) prior to injection.

### **2.2 Isolation and Injection of *Xenopus laevis* Oocytes**

*Xenopus laevis* frogs (from NASCO International, Fort Atkinson, WI and Xenopus Express, Plant City, FL) were housed in static aquarium tanks at 19-21°C with a 12/12 hour light/dark cycle. They were fed fishmeal or frog brittle 2-3 times per week. Before surgery, the frogs were anesthetized. Then a small incision was made in the abdominal wall and a small piece of ovary was excised.

Stage V and VI oocytes were isolated by removing the inner ovarian epithelium layer and theca layers manually with forceps in hypertonic isolation media (108 mM NaCl, 2 mM KCl, 1 mM EDTA, and 10 mM HEPES, pH 7.5), which cause the oocytes to shrink from the encapsulating membranes. Oocytes were then treated with collagenase solution for 10 minutes following isolation to remove the follicular cell layer of the oocytes. Collagenase solution is composed of 0.5 mg/ml of Sigma type IA collagenase in collagenase buffer (83 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES, pH 7.5). The oocytes were removed to incubation medium for injection with cDNAs. Incubation media consists of filtered Modified Barth's solution (MBS; containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO<sub>3</sub>, 10 mM HEPES, 0.82 mM MgSO<sub>4</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub> and 0.91 mM CaCl<sub>2</sub>) that is supplemented with 10mg/l streptomycin, 10,000 units/l of penicillin, 50 mg/l of gentamicin, 90 mg/l theophylline and 220mg/l pyruvate.

The wild type and mutant  $\alpha_1$  glycine receptor subunit cDNAs (1.0 ng/30nl) were injected into the nucleus of oocytes located at the pole of the animal hemisphere. Colman's "blind" method was used to perform for nuclear injection of cDNAs (Colman, 1984). cDNAs were injected using a microdispenser (Drummond Scientific, Broomwall, PA) and needles pulled with a flaming/brown micropipette puller (Sutter Instrument Co., Novato, CA) and cut to a diameter of 10-20  $\mu$ M. Following injections, oocytes were placed singly in 96 well plates (Corning Glass Works, Corning, NY) containing incubation media and incubated at 15°C. Following incubation, electrophysiological recordings were made in the oocytes 1 to 12 days after injection (Figure 5).



**Figure 5. Isolation and injection of *Xenopus laevis* oocytes.** Oocytes were isolated from female *Xenopus laevis* frogs, injected with cDNAs encoding the GlyR  $\alpha 1$  subunit, and then studied using two-electrode voltage clamp electrophysiology.



When expressed in a heterologous system, such as *Xenopus laevis* oocytes, GlyR  $\alpha 1$  subunits can assemble homomERICALLY to form functioning receptors with properties like those of native receptors (Taleb and Betz, 1994). In the accessibility experiments with sulfhydryl specific reagents, wild type GlyR  $\alpha 1$  cDNAs were injected and studied along with following  $\alpha 1$  subunit mutants: I229C, G256C, T259C, V260C, M263C, T264C, S267C, S270C, and A288C. In the crosslinking studies, the following  $\alpha 1$  subunit mutant receptors were injected in addition to the wild type: S267C, A288C, S267C + A288C in a 1:1 ratio, S267C/A288C, S270C/I285C, and M263C/L291C cDNAs.

### **2.3 Electrophysiological Recording**

Electrophysiological recordings were made in the oocytes 1 to 12 days after injection. All experiments were performed at room temperature (20-22°C). Oocytes were placed in a depression in the center of a rectangular chamber (approximately 100  $\mu$ l volume), impaled in the animal pole with two glass electrodes (0.5-10 megaohm) filled with 3 M KCl, and clamped at a voltage of -70 mV using a Warner Instruments OC725C (Hamden, CT) oocyte clamp. The oocytes were perfused with filtered Modified Barth's solution (MBS) containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO<sub>3</sub>, 10 mM HEPES, 0.82 mM MgSO<sub>4</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub> and 0.91 mM CaCl<sub>2</sub>. The perfusion rate was 2.0 ml/minute and controlled by a peristaltic pump (Cole-Parmer Instruments Co., Chicago, IL) through 18-gauge polyethylene tubing (Becton Dickinson, Sparks, MD). The oocytes

were tested for expression by measuring whether a current was elicited by application of glycine dissolved in MBS. Oocytes expressing the injected receptors were used in the following experiments and currents were continuously plotted using a Cole-Parmer chart recorder (Vernon Hills, IL).

Concentration response curves were determined for the wild type and each mutant receptor studied. Concentrations of glycine, usually ranging from 10  $\mu$ M – 1 mM, were applied at 10-15 minute intervals in order of increasing concentration until a plateau was reached. For sensitive receptors, even lower concentrations were tested, and for less sensitive receptors, higher concentrations of glycine were used to reach a plateau. Curves were analyzed using GraphPad Prism as described in chapter 2.7. The concentration of glycine producing the greatest response in the concentration response curves was used to elicit the maximal glycine response in subsequent experiments.

## **2.4 Crosslinking Transmembrane Segments 2 and 3**

Glycine (1 mM) was dissolved in MBS and applied for 20 s (30 s for lower concentrations). Dithiothreitol (DTT) was freshly prepared and dissolved in MBS at a concentration of 1 mM or 10 mM prior to each three-minute application. Mercuric chloride ( $\text{HgCl}_2$ ; 10  $\mu$ M) was prepared from a 1 mM stock in MBS and applied to crosslink (Soskine et al., 2002) for one minute. Strychnine (10  $\mu$ M) was prepared from a 1 mM stock in MBS and applied for 40 s.

### **2.4.1 Reduction and Crosslinking Experiments**

Reduction experiments were performed as follows: 1 mM glycine was applied to the oocytes followed by a washout of 15 minutes. This was repeated twice. Then the oocyte was unclamped during the 1 mM DTT application because DTT affected the bath electrodes or impaling electrodes, causing the oocyte clamp to be unable to maintain the -70 mV potential in the mutant, WT and uninjected oocytes. The oocyte was resealed, and was washed for 15 minutes. Glycine was then reapplied three times with 15 minute washouts with the last response to glycine being measured 45 minutes after DTT. The reduction and crosslinking experiments were performed as follows: Glycine (1 mM) was applied to the oocyte, followed by a 15 minute washout. This was repeated once. This was followed by reduction with 10 mM DTT (oocyte unsealed) and a 15 minute washout. Glycine was reapplied with a 15 minute washout. Then the oocyte was unsealed again during a 1 minute application of 10  $\mu$ M HgCl<sub>2</sub> for crosslinking. Glycine was reapplied with a 15 minute washout. This was followed by a second reduction with DTT and 15 minute washout (as above) and a final application of glycine.

### **2.4.2 Drug Responses in Reduced and Crosslinked Receptors**

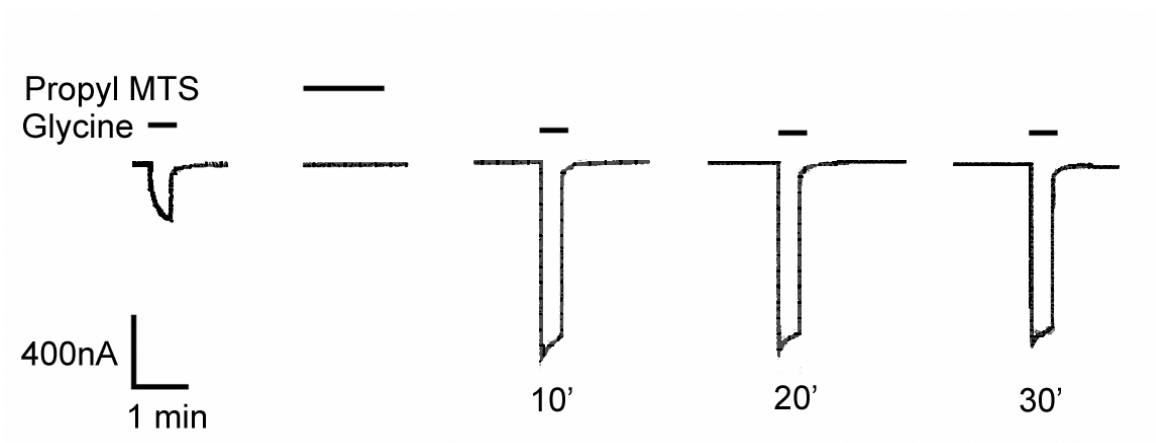
Ethanol (100 mM), octanol (115  $\mu$ M), isoflurane (0.8 mM), and chloroform (2.0 mM) were dissolved in MBS immediately prior to each experiment. Responses to these concentrations of alcohols and anesthetics were tested (following a 1 minute preincubation with the drug alone) on an EC<sub>5-10</sub> of glycine (concentration of glycine eliciting 5-10% of the maximal glycine response), which was determined individually for

each oocyte. S267C/A288C mutants were tested for their drug responses in the crosslinked state before DTT application. After reduction of S267C/A288C with 1 mM DTT, the EC<sub>5-10</sub> concentration of glycine was re-determined for each oocyte, and the drugs were tested in the same manner. The drug responses were measured approximately 45 minutes after reduction to ensure that most receptors were uniformly in the reduced form.

## **2.5 Experiments Using Sulfhydryl-Specific Compounds**

### **2.5.1 Accessibility**

First the EC<sub>5</sub>-EC<sub>10</sub> of glycine (5-10% of the maximal response to 1 mM glycine) was determined for each expressing oocyte. After a 10 minute washout, MTS reagents were applied in either the absence of glycine (closed state) or in the presence of 1 mM glycine (open and desensitized states). Then responses to the initial EC<sub>5-10</sub> of glycine were determined at timepoints 10, 20 and 30 minutes after application of MTS. The percent potentiation of the recorded glycine current over the initial current before the MTS application was evaluated for each oocyte (Figure 6).



**Figure 6. An example tracing of MTS application procedure.** This tracing shows S267C reaction with 50  $\mu$ M propyl MTS. An  $EC_{5-10}$  glycine (5-10% of the maximal response to 1 mM glycine) was determined for each expressing oocyte. The first maximal glycine response is not shown. After a 10 minute washout, propyl MTS was applied in either the absence of glycine (closed state) as shown above, or in the presence of 1 mM glycine (open and desensitized states). The initial  $EC_{5-10}$  of glycine was reapplied at washout times of 10, 20 and 30 minutes after application of MTS.

Wild type and mutant receptors were perfused for 90 s with a 50  $\mu$ M solution of either propyl MTS, hexyl MTS, octyl MTS, decyl MTS, dodecyl MTS, hexadecyl MTS, benzyl MTS or *para*-chloromercuribenzenesulfonate (pCMBS<sup>-</sup>) in either the absence of glycine or in the presence of 1 mM glycine. In testing these differently sized MTS compounds for labeling in the open and the closed states, information about the accessibility and volume of this portion of the binding pocket can be better appreciated. All MTS applications were for 90 s, unless otherwise specified as this gave a steady-state reaction with S267C. MTS compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted in MBS to a final DMSO concentration not exceeding 0.05% (for hexadecyl MTS the final concentration was 0.1%). These concentrations of DMSO did not affect GlyR function. MTS solutions were prepared immediately before application to prevent degradation in all experiments (unless otherwise noted).

For some mutants, the maximal glycine response required a higher concentration of glycine from the concentration response curve data. In these cases, higher concentrations of glycine were used for co-application with MTS reagents. For G256C, T259C, V260C, and A288C, 10 mM glycine was used as the maximum glycine concentration for labeling and to determine the EC<sub>5-10</sub>. For Y410C, 5 mM glycine was used as the maximal glycine concentration. During co-applications of MTS reagents with maximal glycine, the voltage clamp circuit on the oocyte was inactivated to prevent cell damage and run down of the glycine current.

### **2.5.2 Testing for Silent Reaction of MTS**

To detect whether decyl MTS was reacting “silently” with S267C, but producing no observable change in current, the EC<sub>5-10</sub> of glycine was determined, and an application 100 µM decyl MTS (in MBS) was followed by measurement of the glycine response. This was followed by an application of 50 µM propyl MTS (in MBS) and measurement of the glycine response. Responses to the initial EC<sub>5-10</sub> of glycine were measured 10 minutes after each MTS application. We also tested the stability of propyl MTS in room temperature MBS. We observed no change in effectiveness of 1-hour-old 50 µM propyl MTS solutions compared with freshly prepared solutions on S267C.

### **2.5.3 MTS Concentration Response Curves for S267C**

Concentration response curves for propyl and decyl MTS in the presence and absence of glycine were determined for irreversible enhancement of S267C. Different oocytes were used for each concentration ( $n = 4-11$  oocytes per concentration point). For each oocyte (at ten minute intervals): the EC<sub>5-10</sub> of glycine was determined, the MTS compound was applied for 90 seconds, and the original EC<sub>5-10</sub> of glycine was re-applied. Concentrations ranging from 1-1000 µM of propyl MTS and concentrations ranging from 10-300 µM decyl MTS were tested.

#### **2.5.4 Rate of Reaction with MTS in the Presence and Absence of Glycine and/or Anesthetics**

The rate of reaction of propyl MTS (50  $\mu$ M) with S267C was determined in four conditions: 1) no glycine, 2) no glycine plus 0.6 mM isoflurane, 3) 1 mM glycine and 4) 1 mM glycine plus 0.6 mM isoflurane. For conditions 1 and 3, the EC<sub>5-10</sub> of glycine was first determined for each oocyte. After 10 minutes, propyl MTS (50  $\mu$ M) was applied for 15 s in the absence of glycine (10 s applications in the presence of glycine because the cumulative reaction time was shorter). Ten minutes following the propyl MTS application (or 15 min for MTS applications with glycine to allow time for receptor recovery from desensitization), the original EC<sub>5-10</sub> of glycine was re-applied. This procedure was repeated until the glycine response reached a steady-state. For conditions 2 and 4, the application procedure was identical and 0.6 mM isoflurane was co-applied with propyl MTS in the presence and absence of glycine. All MTS and isoflurane solutions were prepared immediately before each application to ensure that a uniform concentration of these compounds reached the oocytes.

Currents were normalized using the following procedure to put each oocyte's response on a scale of 0 (initial current) to 1.0 (steady-state current). For each oocyte, all currents were divided by the initial current. Then 1 was subtracted from all of the values. These normalized responses for each oocyte were fit to a one phase exponential association curve to determine the time and rate constants of each curve. These time constants were then averaged and presented with their standard errors and the second



order rate constants were calculated by dividing the averaged rate constants by the concentration of MTS applied.

The rates of reaction of hexyl and decyl MTS with S267C were determined in the same manner. Ten second applications of 50  $\mu$ M hexyl MTS were applied in the closed state, and 10 s applications of 5  $\mu$ M hexyl MTS (or 1  $\mu$ M decyl MTS) were applied in the presence of 1 mM glycine until a steady state response was reached. Additionally, the reaction rate constant for the reaction of propyl MTS (500  $\mu$ M) with A288C was determined in the presence of 1 mM glycine.

### **2.5.5 Differentiating Reactive Receptor States**

To differentiate the receptor states MTS reacts with, the potentiation by 0.8 mM isoflurane was measured following decyl MTS reaction with S267C receptors in the closed (as a control), desensitized and open/desensitized states. The isoflurane potentiation of the EC<sub>5-10</sub> glycine current was measured for each condition and compared to that of unlabeled receptors. As shown previously, receptors that could be labeled by MTS would have eliminated or reduced isoflurane potentiation (Mascia et al., 2000). Receptors were labeled (90 s) in the closed state (50  $\mu$ M decyl MTS) and open/desensitized state (50  $\mu$ M decyl MTS + 1 mM glycine). Desensitized receptors were labeled after a 10-14 minute application of 1 mM glycine that left only 1-5% of the maximal current activatable. Then the oocyte was washed in MBS (30 s), followed by application of 50  $\mu$ M decyl MTS in MBS to label in either the desensitized or closed

state. For all of the above conditions, the isoflurane potentiation was determined 15 minutes after MTS labeling. Potentiation was calculated by dividing the drug-induced current by the average EC<sub>5-10</sub> glycine-induced currents applied 10 minutes before and after each drug application. For the control, unlabeled receptors, the isoflurane potentiation was determined as above. Isoflurane was dissolved in MBS or glycine solutions immediately prior to each experiment. Samples of bath solutions of isoflurane reaching the oocyte were measured by gas chromatography to have a 50% loss from the prepared vial solutions. Thus, we prepared a vial solution of 1.6 mM to produce a bath concentration at the oocyte of 0.8 mM isoflurane (approximately 2.4 times the anesthetic EC<sub>50</sub>) (Franks and Lieb, 1994).

## **2.6 Chemicals and Reagents**

Glycine was purchased from Bio-Rad (Hercules, CA). All sulfhydryl-specific reagents were purchased from Toronto Research Chemicals (Toronto, Ontario). Isoflurane was purchased from Ohmeda Caribe Inc. (Liberty Corner, NJ) and Marsam Pharmaceuticals, Inc. (Cherry Hill, NJ). Ethanol was purchased from AAPER Alcohol and Chemicals Co. (Shelbyville, KY). Dithiothreitol, mercuric chloride, strychnine, octanol, chloroform and all other electrophysiological reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

## **2.7 Statistical Analysis**

Statistical analyses were performed using GraphPad Prism 3.02 and 4.0 (San

Diego, CA). The software was used to fit concentration response curves with non-linear regression curve fitting for a sigmoidal dose-response curve with a variable slope. Wild type and mutant EC<sub>50</sub> and Hill coefficients were compared using one-way ANOVA with the Dunnett's post-test. Additionally, it was used to define significance of the glycine responses measured, either following MTS reagent applications or responses modulated by drugs, versus the control EC<sub>5-10</sub> glycine responses using the paired Student's *t*-test. For the rate of reaction experiments, the response of each oocyte was fit to a one phase exponential association curve to determine the time and rate constants. These values were averaged and presented with their standard errors.

## **2.8 Molecular Volume Calculations and Modeling**

All molecular modeling was carried out by Dr. James R. Trudell of Stanford University School of Medicine who was a collaborator on this project, a co-author of the resulting publications and a member of my thesis committee. The volumes of the MTS reagents, the volumes of the corresponding alkyl thiols that functionally react with the substituted cysteine residue, and the volumes of the amino acids cysteine and serine were calculated using Spartan 5.0 (Wavefunction, San Diego, CA).

Molecular modeling of the glycine receptor transmembrane region was conducted as previously described (Trudell and Bertaccini, 2004). A model of the four transmembrane segments of a glycine receptor was built by threading the primary sequence of GlyR  $\alpha 1$  over a template of a four-helix bundle found in the high-resolution structure of the cytochrome C oxidase (Protein Data Bank code 20CC). An initial

constraint on the model was that amino acid residues known to modulate anesthetic potency were in direct proximity to one another [I229 (TM1), S267 (TM2), and A288 (TM3)]. A second set of constraints was that the pore-facing and lipid-facing residues identified in the literature should have appropriate positions. This model was used to examine the proximity of S267C and A288C, and also to predict nearby residues to S267, as described below.

The model was used to examine whether crosslinking between S267C and A288C was possible. This molecular model positioned S267C and A288C in proximity to form a disulfide bond. Residues S267 and A288 were replaced with cysteines and an S-S bond was formed between them. All backbone atoms (C, Ca, N, O) were tethered to their initial positions with a force constant of  $100\text{kcal}/\text{\AA}^2$  and the structure was subjected to restrained molecular mechanics optimization with the CFF91 force field using Insight II (v 2000.1, Accelrys, San Diego, CA).

Additionally, the model revealed that other residues in TM2 might be in proximity to S267 and could be accessible to MTS reagents (G256, T259, V260, M263, T264, S270). The positioning of hexyl MTS was based on forming the disulfide bond and then re-optimizing the GlyR model with harmonic restraints ( $100\text{ kcal}/\text{\AA}^2$ ) on all the backbone atoms of the subunit to illustrate a likely orientation and show the scale of the molecule relative to the subunit.

### 3.0 Orientation of Transmembrane Segments 2 and 3

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Lobo IA, Trudell JR, Harris RA. Cross-linking of glycine receptor transmembrane segments two and three alters coupling of ligand binding with channel opening. *J Neurochem.* 2004 Aug;90(4):962-9.

#### 3.1 Introduction

Strychnine sensitive glycine receptors (GlyRs) are a member of a family of ligand-gated ion channels. Receptors of this family are arranged as five subunits surrounding a central pore with each subunit composed of four alpha helical transmembrane segments (TM1-TM4) (Miyazawa et al., 2003; Rajendra et al., 1997). Miyazawa et al. recently extended the resolution of the homologous *Torpedo* acetylcholine receptor to 4 Å, showing an inner ring of TM2 alpha helices line the channel pore with a second outer ring formed by the 15 alpha helices of TM1, 3 and 4 (Miyazawa et al., 2003).

Sites of alcohol and volatile anesthetic action on the glycine receptor (and the homologous GABA<sub>A</sub> receptor) have been identified: in TM1 (I229), TM2 (S267), as well as a residue in TM3 (A288) (Greenblatt and Meng, 1999; Jenkins et al., 2001; Mihic et al., 1997; Ueno et al., 1999; Wick et al., 1998; Yamakura et al., 1999; Ye et al., 1998b). These amino acids have been hypothesized to line a binding pocket for alcohols and volatile anesthetics between the four transmembrane segments of each subunit (Jenkins et al., 2001; Mascia et al., 2000; Yamakura et al., 2001). Though the pore lining residues of TM2 have been defined by cysteine substitution and labeling in this family of proteins (Horenstein et al., 2001; Xu and Akabas, 1996; Zhang and Karlin, 1998), the contact points between the non-pore lining TM2 residues that may face TM1, 3 or 4 are undetermined. Considering that these amino acids are distant from one another in the primary amino acid sequence and that there is no complete crystal structure available for the glycine receptor, we were interested in determining how these amino acids were arranged.

Currently, there are two pieces of experimental evidence that suggest S267 and A288 may be near one another from data gathered in homologous receptors. First, Wick et al. exchanged amino acids in TM2 and TM3 between the homomeric GABA  $\rho$ 1 and GlyRs to alter alcohol cutoffs of these two receptors. The alcohol cutoff is the point at which increasing the length of the alkyl chain of a primary alcohol no longer produces an increase in potency. The homomeric GlyR  $\alpha$ 1 has an alcohol cutoff at decanol, while the GABA  $\rho$ 1 receptor cutoff is at heptanol, suggesting that the GABA  $\rho$ 1 alcohol binding pocket is smaller than that of the GlyR. When homologous positions of GABA  $\rho$ 1 (I307

and W328) were converted to their smaller glycine receptor counterparts (S267 and A288), the alcohol cutoff of the single mutant receptors increased from 7 to 9. Mutation of both of these amino acids to I307S/W328A increased the alcohol cutoff to be dodecanol or higher, suggesting that both residues were a part of a single alcohol binding cavity (Wick et al., 1998). Secondly, Jenkins et al. studied homologous positions of the GABA<sub>A</sub> receptor, S270 and A291, and replaced them with the bulky amino acid tryptophan. The S270W single mutant was insensitive to the anesthetics isoflurane and halothane, but retained sensitivity to the smaller anesthetic chloroform. Meanwhile, when both were replaced to create a S270W/A291W double mutant, the receptor was insensitive to chloroform as well as isoflurane and halothane (Jenkins et al., 2001). Here, we tested for a direct association of the GlyR  $\alpha 1$  amino acids positions S267 and A288 to determine the orientation of TM2 and TM3.

Crosslinking has been used for decades to gather structural information about proteins. Existing and introduced cysteines have been crosslinked to determine near-neighbor relationships and associations of proteins, orientations of interactions as well as the activity, folding and three-dimensional structures of diverse proteins. In the GABA<sub>A</sub> receptor, crosslinking was used recently to identify extracellular domain residues that interact with the TM2-TM3 linker loop to couple agonist binding and gating (Kash et al., 2003), and to determine intersubunit TM2 segment contact points (Horenstein et al., 2001). Other studies have explored crosslinking between transmembrane helices in engineered and wild type helical bundle proteins, such as keratin, cyclic nucleotide-gated channels and aspartate receptors (Chervitz and Falke, 1995; Fraser et al., 1988; Matulef

and Zagotta, 2002; Regan et al., 1994). Disulfide crosslinking between adjacent alpha helices occurs when the residues are on opposing faces of the helices (Lee et al., 1995; Soskine et al., 2002) and have C $\alpha$ -C $\alpha$  distances less than 10 Å (Yang et al., 1996).

In the present study, we tested for crosslinking between cysteines introduced at two glycine receptor positions (S267 and A288) known to be involved with alcohol and inhaled anesthetic action. The S267C/A288C double mutant had different characteristics from the wild type (WT) receptor. Its current decreased with repeated applications of glycine, and it displayed tonic activity in the absence of neurotransmitter. These characteristics were eliminated with the application of the reducing agent dithiothreitol and regained after application of mercuric chloride. Mercuric chloride is a crosslinking agent that reacts with vicinal pairs of cysteines to form an intermolecular mercury-linked dimer, even in transmembrane regions with a low dielectric environment (Soskine et al., 2002).

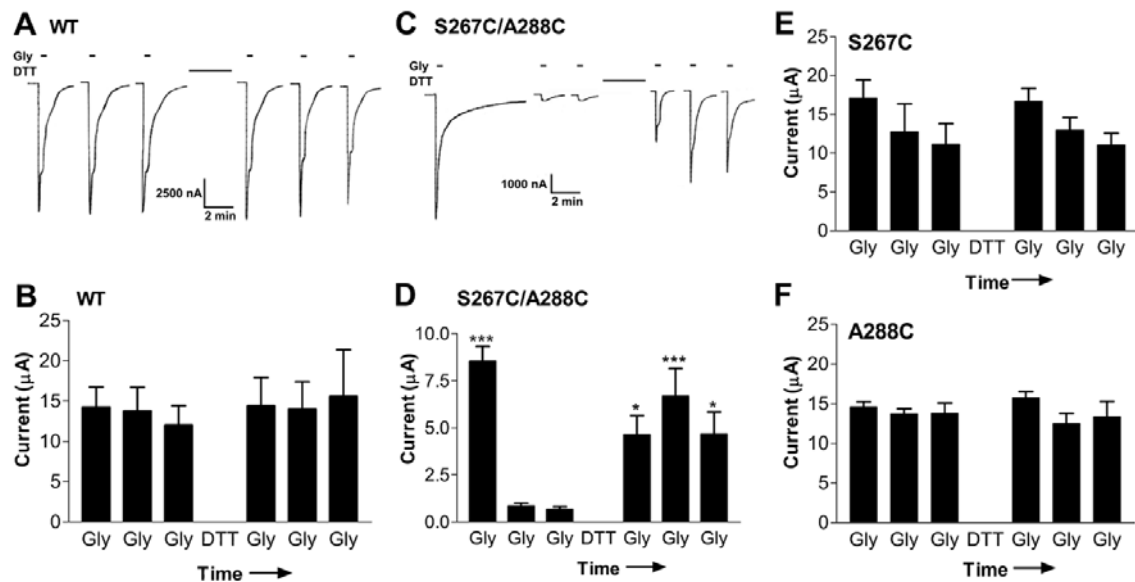
Our results indicate that a disulfide bond had formed between these two introduced cysteine residues to crosslink transmembrane segments two and three. This is strong evidence that S267 and A288 are near-neighbors in the tertiary glycine receptor structure and that these amino acids both could contribute to a binding pocket for alcohols and volatile anesthetics. Some of this work has been presented previously in abstract form (Lobo et al., 2003).



## **3.2 Results**

### **3.2.1 Effect of Reduction on Glycine Currents in Wild Type and Mutant GlyRs**

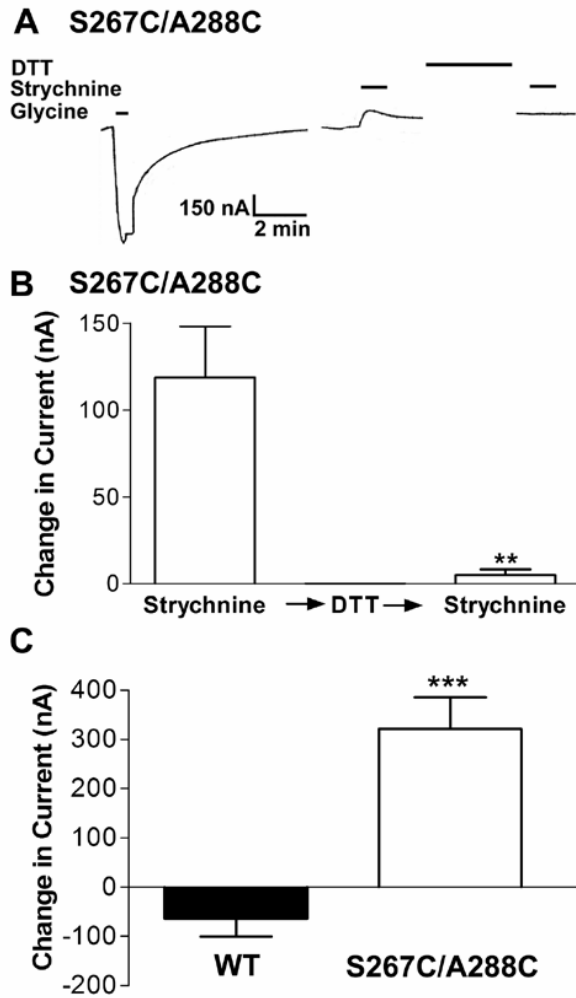
In wild type glycine receptors, repeated exposures to 1 mM glycine elicited similar currents over time, and a three minute application of 1 mM DTT produced no significant change in the receptor function (Figure 7A and 7B). Unlike the wild type, exposure to glycine (1 mM) caused a run-down of the glycine response in the S267C/A288C mutant. This run-down was observed for 30 minutes after the first glycine application. Following application of 1 mM DTT, the response of S267C/A288C to glycine recovered significantly (Figure 7C and 7D). The single mutants, S267C and A288C were tested as controls and displayed responses similar to the wild type (Figure 7E and 7F). Oocytes co-injected with a 1:1 ratio of S267C + A288C did not show a current run-down and were similar to the wild type, indicating that intersubunit crosslinking was not occurring ( $n = 8$ , data not shown).



**Figure 7. Reduction with dithiothreitol has no effect on wild type, S267C and A288C glycine receptors, but increases the response of S267C/A288C receptors to subsequent applications of glycine.** Glycine (1 mM, 20 s) was applied (at 15 minute intervals) three times before and three times after a three minute application of DTT (1 mM) to oocytes expressing wild type or mutant glycine receptors. A) A tracing of a single wild type response shows no change in current after reduction. Oocytes were unclamped during DTT treatments, so this portion of the tracing is not shown. B) Mean currents in the wild type receptor show no change with repeated applications of glycine and no change after reduction. C) A tracing of a single oocyte expressing S267C/A288C shows that exposure to 1 mM glycine resulted in decrease in subsequent glycine responses, and that DTT reduction causes an increase in the glycine response. D) The mean currents in the S267C/A288C receptor show the decreasing current elicited by glycine before reduction, and recovery of current after reduction. E) and F) Mean currents in the S267C and A288C single mutants also show no change in current with repeated applications of glycine and no change after application of DTT. Mean values  $\pm$  SEM are shown for  $n = 5-13$  oocytes per condition from 2-5 batches of oocytes. \*,  $p < 0.05$ , \*\*\*,  $p < 0.001$  as compared to final glycine response pre-reduction by one-way analysis of variance followed by Dunnett's post test.

### **3.2.2 Effect of Reduction on GlyR $\alpha 1$ (S267C/A288C) Leak Current, Tonic Activity and Baseline Current**

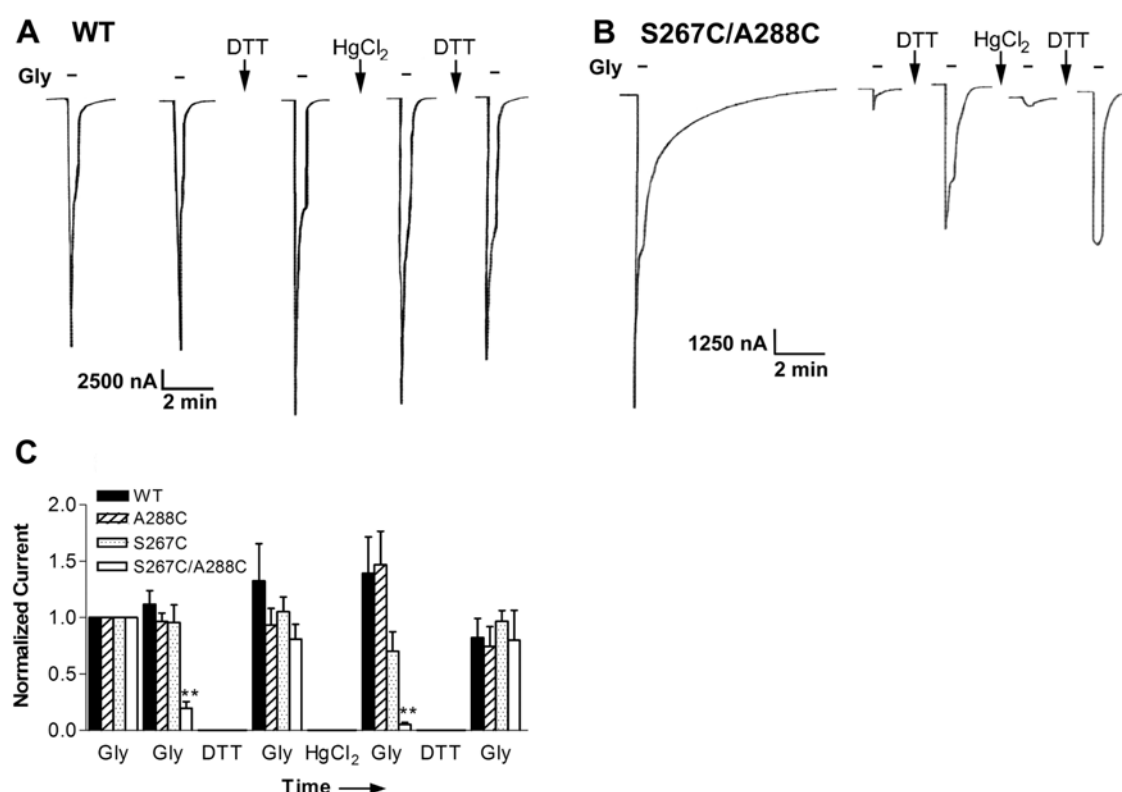
Immediately after clamping, a large, inward leak current, sensitive to strychnine, was apparent in the majority of GlyR  $\alpha 1$ (S267C/A288C)-expressing oocytes tested. This current, never seen in the wild type receptor, declined immediately from the time of clamping to reach a stable baseline within 5-15 minutes. When a stable baseline was reached, all experiments were performed. Wild type glycine receptors do not respond to the channel antagonist strychnine in the absence of glycine. The S267C/A288C mutant; however, displayed a tonic current after reaching a stable baseline that was reduced by strychnine, which closed the open channels. This resulted in a decrease of the tonic inward current, suggesting that some channels were open in the absence of glycine (Figure 8A). The strychnine effect on S267C/A288C receptors did not depend on prior activation with glycine. After reduction with 1 mM DTT, strychnine no longer had a significant effect on the mutant receptors (Figure 8A and 8B). After reduction, there was no leak current observed upon clamping. Also, the baseline shifted, indicating that the spontaneous inward leakage current was reduced in the mutant receptor and that the mutant channels had closed. This shift in baseline was significantly different from the wild type receptors (Figure 8C).



**Figure 8. Effects of reduction on S267C/A288C tonic activity and baseline shift following reduction.** A) Unlike wild type receptors that do not respond to applications of strychnine, S267C/A288C receptors close with a 10  $\mu$ M strychnine application (40 s). The strychnine response occurred both without prior activation with glycine or after activation with glycine (as shown above). After reduction with DTT (1 mM, 3 min), strychnine no longer had an effect. B) The mean decrease in the tonic inward current of S267C/A288C receptors by 10  $\mu$ M strychnine before and after reduction ( $n = 9$  oocytes per condition from 3 batches of oocytes). \*\*,  $p < 0.01$  as compared to the effect before reduction by the Student's  $t$  test. C) The baseline current shifted after reduction in the S267C/A288C mutant and appeared as a decrease in inward current. The change in baseline of the wild type was compared to S267C/A288C. Mean values  $\pm$  SEM are shown for  $n = 14$  wild type oocytes from 5 batches of oocytes, and from  $n = 34$  mutants from 16 batches of oocytes. \*\*\*,  $p < 0.001$  as compared to the wild type using the Student's  $t$ -test.

### **3.2.3 Effects of Reduction and Crosslinking on Wild Type, S267C, A288C and S267C/A288C Glycine Receptors**

We also asked if the mutant receptor could be cycled between its aberrant and wild type characteristics by reduction with DTT, followed by re-crosslinking with HgCl<sub>2</sub> (Soskine et al., 2002) and then a second application of DTT. This experiment tested the reversibility of covalent bond formation between these two amino acids in the mutant receptor. In the wild type receptors there was no significant change in current (elicited by 1 mM glycine) after applications of either DTT or HgCl<sub>2</sub> (Figure 9A and 9C). For either S267C or A288C, there was no significant change in mean current after applications of either DTT or HgCl<sub>2</sub>, though both showed variable responses like the WT (some oocytes showed a decrease or an increase in current, while others showed no change) after the HgCl<sub>2</sub> applications (Figure 9C). The current of the S267C/A288C mutant receptor was altered significantly after applications of DTT or HgCl<sub>2</sub>, with DTT causing a significant increase in the receptors' response and HgCl<sub>2</sub>, causing a significant decrease in receptor function. These functional changes were measured 15 minutes after the DTT or HgCl<sub>2</sub> applications (Figure 9B and 9C).



**Figure 9. Effects of reduction and crosslinking on WT, A288C, S267C and S267C/A288C glycine receptors currents.** Two applications of glycine (1 mM, 20 s) were applied at 15 minute intervals, followed by reduction with DTT (10 mM, 3 min.), glycine (1 mM), crosslinking with HgCl<sub>2</sub> (10  $\mu$ M, 1 min.), glycine (1 mM), a second reduction step with DTT (10 mM, 3 min.), and a final test with glycine (1 mM). A) This tracing of the glycine response in the WT receptor shows that the current does not change significantly after reduction and crosslinking. B) A tracing from the S267C/A288C mutant shows that reduction increases the glycine response, mercuric chloride nearly eliminates the current induced by 1 mM glycine, and that a subsequent reduction step again increases the glycine response. C) The normalized mean currents  $\pm$  SEM are shown for the WT, A288C, S267C and S267C/A288C receptors. Currents were normalized to 1.0 by dividing the currents of the wild type and mutant receptors by the current induced by the initial glycine application. The data represents mean currents from  $n = 5-6$  oocytes per condition from 2-4 batches of oocytes. One-way analysis of variance followed by the Dunnett's post test was used to determine differences in the mutant glycine responses in comparison to the respective wild type response (\*\*,  $p < 0.01$ ).

### **3.2.4 Effects of Alcohols and Volatile Anesthetics on Wild Type and Mutant Glycine Receptors**

The effects of two alcohols (ethanol and octanol) and two volatile anesthetics (isoflurane and chloroform) were tested on the wild type, S267C, A288C, and S267C/A288C receptors. The EC<sub>5-10</sub> of glycine was determined for each oocyte. Responses were tested after a one minute pre-incubation of the drug, using the EC<sub>5-10</sub> glycine. The single S267C mutation reduced or eliminated all drug responses tested except that of isoflurane. Meanwhile, the A288C mutation reduced or eliminated all drug responses tested except chloroform. The double mutant, S267C/A288C, reduced or eliminated the responses of all four drugs in comparison to the wild type receptors. The mean responses of the wild type and mutant receptors to these four drugs and the average EC<sub>5-10</sub> glycine values of the receptors are presented in Table 1.

**Table 1**

Glycine EC<sub>5-10</sub> and alcohol and volatile anesthetic responses of the WT glycine receptors and the cysteine substitution mutants.

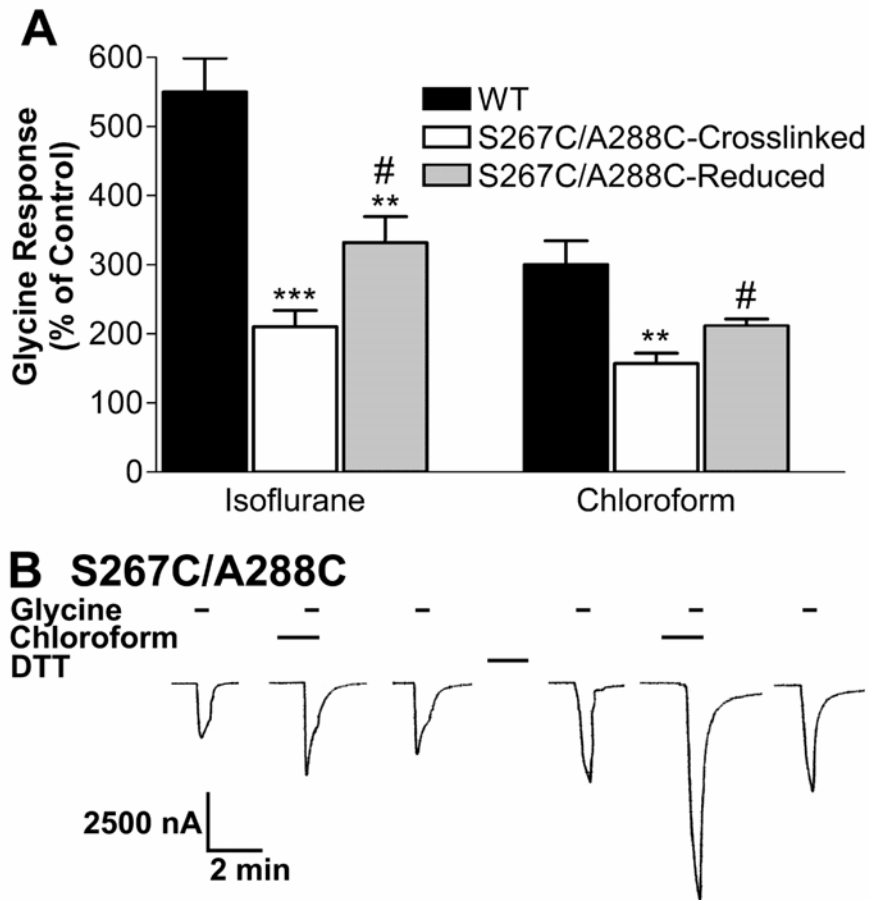
Glycine Receptor	EC <sub>5-10</sub> (μM)	Percent Potentiation			
		Ethanol	Octanol	Isoflurane	Chloroform
WT	63 ± 4	80 ± 10	170 ± 20	450 ± 50	200 ± 30
S267C	43 ± 6	0 ± 6 **	90 ± 30 *	520 ± 150	10 ± 10 **
A288C	230 ± 10 **	-10 ± 10 **	30 ± 20 **	150 ± 30 **	190 ± 30
S267C/A288C	95 ± 10	10 ± 4 **	20 ± 7 **	110 ± 20 **	60 ± 10 **

Percent potentiation of EC<sub>5-10</sub> glycine responses to ethanol (100 mM), octanol (115 μM), isoflurane (0.77 mM) and chloroform (2.0 mM) are expressed as a mean ± SEM of 4 to 28 oocytes per condition from 2-17 batches of oocytes. S267C/A288C receptors were tested for their responses here after application of maximal glycine and EC<sub>5-10</sub> glycine determination in the uniform crosslinked state. \*  $p < 0.05$ , \*\*  $p < 0.01$ ; significantly different from wild type receptors by one-way ANOVA with Dunnett's post test.



### **3.2.5 Effects of Volatile Anesthetics on S267C/A288C Glycine Receptors Before and After Reduction**

To determine whether reduction of the S267C/A288C receptors altered the drug responses, the drugs were tested approximately 45 minutes after application of 1 mM DTT while the receptors were in a uniformly reduced state. After reduction, the  $EC_{5-10}$  glycine was re-determined for each oocyte (average =  $40 \pm 15 \mu\text{M}$ ), and the drugs were tested. The two alcohols still had no effect (data not shown), but the two anesthetics now potentiated the glycine response. The response of the reduced mutant to isoflurane was significantly greater than the response of the crosslinked S267C/A288C receptors, but did not recover to the level seen in the wild type. Additionally, the response of the reduced S267C/A288C receptors to chloroform was significantly larger than that of the crosslinked receptors and recovered to a response similar to that of the wild type receptors (Figure 10).



**Figure 10. Effects of volatile anesthetics on the wild type glycine receptor and drug effects on S267C/A288C before and after reduction.** A) Isoflurane (0.77 mM) and chloroform (2.0 mM) both potentiate the wild type (*black bars*) response to EC<sub>5-10</sub> glycine. These effects are smaller in the crosslinked S267C/A288C mutant (*white bars*). After reduction with 1 mM DTT (3 min), the max glycine response and EC<sub>5-10</sub> glycine was re-determined to test the drugs a second time. Post-reduction, both isoflurane and chloroform potentiated the glycine response to a greater extent than before (*gray bars*). Mean values  $\pm$  SEM are shown for  $n = 4-7$  oocytes per condition from at least 2 batches of oocytes. \*\*,  $p < 0.01$ , \*\*\*  $p < 0.001$ , as compared to wild type potentiation and #,  $p < 0.05$  as compared to crosslinked receptor potentiation, Student's  $t$ -test. B) This example tracing of the S267C/A288C mutant shows the effect of chloroform on the EC<sub>5-10</sub> glycine response in the crosslinked and reduced states in a single oocyte.

### 3.2.6 Additional Double Cysteine Mutants

Two additional double cysteine mutants were tested. In both cases, the pairs of amino acids chosen to mutate were predicted by the homology model and/or evidenced by the substituted cysteine accessibility method to face the water-filled, drug-binding cavity at the center of the four transmembrane segments of each  $\alpha 1$  subunit. The pairs were either an alpha helical turn up or down from the S267/A288 pair. GlyR  $\alpha 1$ (M263C/L291C) is a turn more cytoplasmic, and GlyR  $\alpha 1$ (S270C/I285C) is a turn toward the extracellular space. Oocytes injected with S270C/I285C ( $n = 26$ ) and M263C/L291C ( $n = 36$ ) did not show any response to applications of 1 or 10 mM glycine. Following application of DTT (1 mM or 10 mM), the S270C/I285C ( $n = 7$ ) and M263C/L291C ( $n = 16$ ) receptors still showed no response to glycine. The GlyR  $\alpha 1$ (S270C/I285C) and GlyR  $\alpha 1$ (M263C/L291C) receptors tested were both unresponsive to glycine. To speculate, these receptors may not have folded properly and were not expressed. It is also possible that disulfide bonds may have formed, but they are stabilizing a non-functional form of the receptor. However, this is unlikely because dithiothreitol did not restore function in either mutant.

### 3.3 Discussion

These results suggest that an intrasubunit disulfide bond forms between S267C and A288C in GlyR  $\alpha 1$ (S267C/A288C) receptors. These two amino acids can covalently react with one another to link transmembrane segments two and three. Disulfide bond formation can occur spontaneously, in the absence of additional oxidizing or crosslinking agents, to change the receptor's characteristics. Reduction of the disulfide bond with dithiothreitol largely restores receptor function to that of wild type. Also, these cysteines can be crosslinked again with an application of mercuric chloride, which adds a 4 Å bridge between the two cysteines.

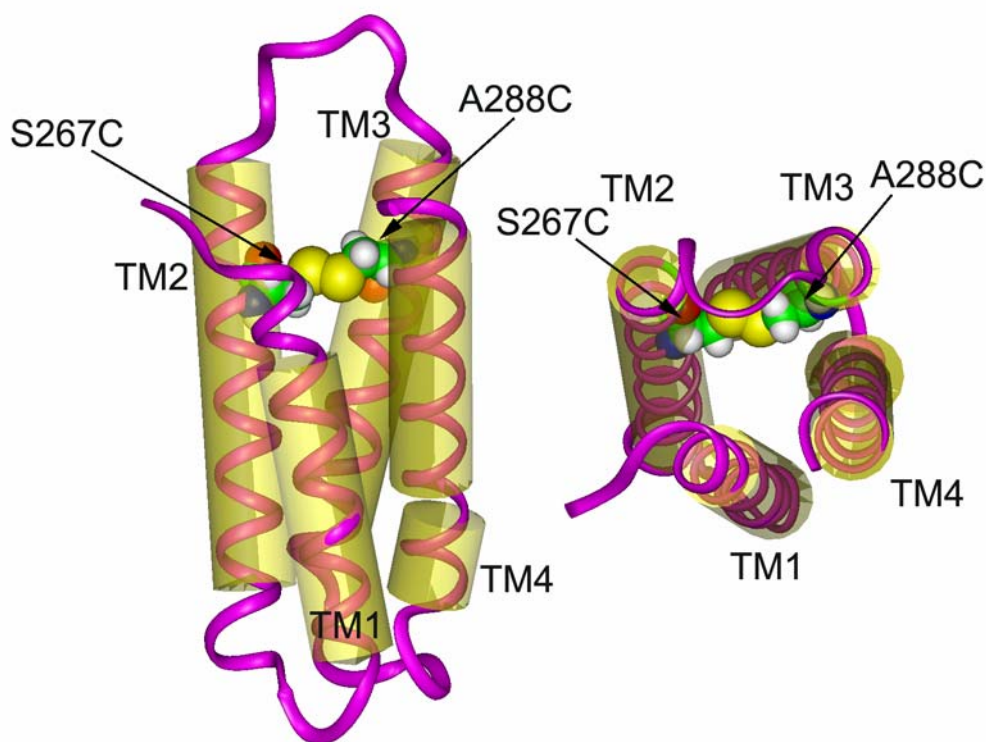
One possibility is that disulfide bonds may form between S267C and A288C during protein folding and processing in the oxidizing environment of the endoplasmic reticulum. However, considering that one of the characteristics of this double mutant is a decrease in the current induced after an application of glycine, it seems likely that some disulfide bonds are forming during the process of channel gating. Support for this hypothesis includes evidence that in the GABA<sub>A</sub> receptor the presence of GABA increases accessibility to amino acids in TM3, indicating movement of TM3 with channel gating (Williams and Akabas, 1999). Also, in the glycine receptor, sulfhydryl-specific reagents react with the glycine receptor single cysteine substitution mutant A288C in the open state of the receptor, but not in the closed state (Harris et al., 2003; Lobo et al., 2004a). These results indicate that a conformational change occurs with channel gating to enlarge a water-filled, intrasubunit cavity and place residue 288 in the putative drug-binding cavity facing S267 (Lobo et al., 2004a). This movement could place S267C and

A288C in proximity to form a disulfide bond. A recent abstract suggests a very low level of crosslinking using oxidizing agents between cysteines introduced into proximate positions (S270C/V292C) of the GABA<sub>A</sub> receptor (Bali and Akabas, 2003), which may generalize our findings in the glycine receptor to the rest of the ligand-gated ion channel family.

Intersubunit crosslinking has been demonstrated between TM2 segments in the GABA receptor (Horenstein et al., 2001). To further test our hypothesis that S267C and A288C faced a common pocket within each subunit, rather than facing the interface and forming intersubunit crosslinks, we compared the behavior of S267C/A288C double mutants with oocytes co-injected with a 1:1 ratio of S267C + A288C cDNAs. The co-injected single mutant subunits behaved like the wild type rather than the S267C/A288C double mutant, indicating that intersubunit crosslinking was not occurring. This supports our model that S267C and A288C face a common intrasubunit pocket.

Conformational analysis of disulfide bridges in high resolution crystal structures indicate the distance between the alpha carbons nearest to the sulfur atoms of covalently-bound cysteines range from 4.6 to 7.4 Å (Thornton, 1981). Analysis of 351 disulfide bridges showed the most common distance is approximately 5.6 Å with a C $\alpha$ -C $\beta$ -S (C-C-S) bond angle of 114 degrees (Petersen et al., 1999). From our molecular model of the glycine receptor transmembrane region, S267C and A288C are positioned at the interface between TM2 and TM3. The residues are in close enough proximity to form a right-handed disulfide bond with a distance and angle comparable to those above. In this model, the distance between the alpha carbons of S267 and A288 is 7 Å and the C-C-S

bond angle is 112 degrees (Figure 11). The two residues are nearer to one another than the  $\sim 10$  Å separation previously reported for the GABA<sub>A</sub> receptor (Bali and Akabas, 2004).



**Figure 11. Model of the disulfide bond formed between S267C and A288C of the GlyR  $\alpha$ 1(S267C/A288C) receptor.** Our data indicates disulfide bond formation in the absence of oxidizing agents between introduced cysteines at positions S267 and A288. This molecular model of the glycine receptor transmembrane regions illustrates this disulfide bond. The distance between the alpha carbons of S267 and A288 is 7 Å and the C-C-S bond angle is approximately 112 degrees.

Endogenous disulfide bonds necessary for glycine receptor function are located in the extracellular domain of the receptor (Rajendra et al., 1997; Rajendra et al., 1995). These disulfide bonds must be well protected because the concentrations of DTT used in our experiments did not alter the wild type glycine receptor function. Others have also noted that exposure of wild type GlyRs and GABA<sub>A</sub> receptors to DTT had no significant effect on current magnitude following washout (Horenstein et al., 2001; Kash et al., 2003; Lynch et al., 2001). Also, there is an endogenous cysteine in TM3 (C290) of the wild type and mutant receptor subunits. There may be a very low level of crosslinking between S267C and C290C because the S267C single mutant showed a non-significant trend in responses similar to S267C/A288C. Overall, because the currents of S267C were statistically similar to wild type, C290 does not seem to be involved in crosslinking with the cysteine introduced in TM2 and other free cysteines in the wild type to any significant extent.

Interestingly, all four of the alcohols and anesthetics tested had eliminated or reduced effects in the S267C/A288C mutant. The simplest explanation for the alcohol data is that alcohol potentiation for the glycine receptor is abolished by the cysteine substitutions independent of formation of disulfide bonds. It is not surprising that reduction cannot restore the wild type effects of alcohols in the double mutant, since the single mutant S267C does not respond to ethanol, and A288C responds to neither ethanol nor octanol. In contrast, after reduction with dithiothreitol, the two volatile anesthetics tested were able to potentiate the S267C/A288C receptors. Isoflurane and chloroform both cause smaller potentiations than the wild type receptors when the S267C/A288C



receptors are crosslinked. After reduction, the chloroform potentiation of S267C/A288C recovered to be similar to WT and the potentiation by isoflurane increased. From these data, it is clear that the disulfide bond is interfering with allosteric modulation by anesthetic drugs on this region of the receptor. One possibility is that reduction of the disulfide bond removes this obstruction from the binding site and increases the volume of the drug-binding cavity to allow the anesthetics to stably bind and cause receptor potentiation.

Natural disulfides serve to stabilize protein structure by decreasing the degrees of freedom of movement (or entropy) of the unfolded state, forcing the equilibrium to the folded state (Wetzel, 1987). Likewise, introduced disulfide bridges have been shown to result in increased stability of mutant proteins (Matsumura et al., 1989). In the case of the S267C/A288C mutant, crosslinking the transmembrane segments to one another results in a functioning protein with an unnaturally limited flexibility. In particular, the region of TM2 where S267 is located has been demonstrated to have high flexibility in nuclear magnetic resonance experiments with TM2 peptides (Yushmanov et al., 2003). Because this region plays a role in conducting the signal of agonist binding to the TM2-3 linker and into TM2 (Kash et al., 2003), it makes sense that our introduced disulfide bond produces changes in channel function.

Restriction of the dynamic movement of TM2 by covalently linking it to TM3 changes channel properties. Wild type glycine receptors exist in multiple receptor states: the unliganded closed state, and many liganded open and desensitized states. The normal dynamics between these receptor states are disturbed by the double bond between S267C

and A288C, and this is noticeable at the whole cell level. As the population of S267C/A288C mutant channels open with the first application of glycine, they take much longer to close than the wild type receptors, indicating the difficulty in moving from one channel conformation to another. Additionally, further applications of glycine produce minimal glycine responses in the mutant receptors, indicating that they may be preferentially stabilized in the desensitized state or "frozen" in the resting state, where they are not responsive to glycine. These characteristics are eliminated with reduction, which shows that once the restrictive disulfide bonds are broken, the channels can behave in a more similar manner to the wild type channels.

These data demonstrate the orientation and near-neighbor proximity of S267 and A288 because of the ability of these two amino acids to form a disulfide bond. These data locate the vertical position of TM2 with respect to TM3 and shows that they face one another. Most importantly, disulfide bonding between these two introduced cysteines provides insight regarding the location and role of the TM2-TM3 interface.

## 4.0 Transmembrane Segment 1

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Lobo IA, Mascia MP, Trudell JR, Harris RA. Channel gating of the glycine receptor changes accessibility to residues implicated in receptor potentiation by alcohols and anesthetics. *J Biol Chem*. 2004 Aug 6;279(32):33919-27. Epub 2004 May 28.

### 4.1 Introduction to Transmembrane Segment 1

The secondary structure of transmembrane segment 1 has been the most difficult to ascertain of the four transmembrane segments. Though it has been known that TM1 is a hydrophobic segment with a length of 20 amino acids, the structure has been controversial. Evidence has suggested it is an alpha helix, beta sheet, and a mixture between the two.

Results using the substituted-cysteine accessibility method in the acetylcholine receptor had an irregular pattern of exposure that did not correspond to either an alpha helix or a beta sheet (Akabas and Karlin, 1995). TM1 showed accessibility in the most

extracellular portion and this region of TM1, with TM2, was hypothesized to contribute to the channel pore (Akabas and Karlin, 1995). Likewise, the pattern of labeling with several with lipophilic photoactivable reagents showed that labeled amino acids had an irregular pattern, and this pattern was also inconsistent with either an alpha helix or a beta sheet (Barrantes, 2003; Blanton and Cohen, 1994). Researchers, have for these reasons, interpreted TM1 as having a substantial amount of non-helical structure in addition to kinks from an evolutionarily conserved proline, present in all members of the LGIC family (Barrantes et al., 2000). More recently, using different hydrophobic, photoreactive probes, Blanton et al. found the structure of TM1 was still not definitive, but could be a distorted alpha helix or beta sheet (Blanton et al., 1998b). In experiments using limited proteolysis of the glycine receptor coupled with mass spectrometry, cleavage sites were noted in TM1 (Leite et al., 2000). The authors suggested these short fragments were more consistent with a beta sheet structure (Leite et al., 2000; Leite and Cascio, 2001).

The 4 Å nicotinic acetylcholine receptor cryo-electron microscopy structure provided perhaps the best data to date to define the secondary structure of TM1 (Miyazawa et al., 2003). Here, TM1 was shown to be an alpha helix, and part of a “classical” four-alpha-helical bundle. TM1, TM3 and TM4 formed an outer ring of fifteen helices surrounding, but separated by water from, the TM2 inner ring of helices (Miyazawa et al., 2003). Additionally, a consensus of 10 secondary structure prediction methods has indicated that TM1 and the other three TM segments are all alpha helical segments (Bertaccini and Trudell, 2002).

In terms of contributing to an alcohol and volatile anesthetic binding site, one position in TM1, I229C, has been implicated as important for volatile anesthetic action. It is, to date, the least characterized of the three known positions critical for alcohol/anesthetic action (I229, S267 and A288). The first evidence that I229 was important for anesthetic action was published by Greenblatt and Meng, who tested a number of TM1 single mutants that converted the original amino acid to the corresponding GABA rho amino acid (Greenblatt and Meng, 1999). They tested the effect of halothane on these mutants, and found that the I229F mutant was not potentiated by halothane. The second reference that mentions this position is by Jenkins et al. who studied the homologous site in the GABA<sub>A</sub> receptor, L232 (Jenkins et al., 2001). They found the L232F mutant was insensitive to halothane, but still sensitive to isoflurane, and found that introducing a larger amino acid L232W caused the receptor to be insensitive to both halothane and isoflurane.

As it is known that there are some conformational changes occurring with channel gating in TM1 of the acetylcholine receptor (Akabas and Karlin, 1995; Zhang and Karlin, 1997), we tested whether neurotransmitter binding changed accessibility to this specific position in the glycine receptor using the substituted-cysteine accessibility method (Karlin and Akabas, 1998). Since I229 has been implicated to be critical for alcohol and anesthetic action, it is an attractive target for mutagenesis and probing with MTS reagents. Here, site-directed mutagenesis was used to substitute a cysteine for the native isoleucine to make an I229C mutant. The I229C receptor was probed using MTS reagents of two lengths. Propyl MTS and decyl MTS were tested for reaction at the

introduced cysteine in the presence and absence of glycine. Then, the effects of ethanol, octanol, isoflurane and chloroform were tested on the mutant and compared to the WT. Additionally, using an experimental strategy used previously for S267C (Mascia et al., 2000), the ability of propyl MTS to block further potentiation of I229C by volatile anesthetics was determined.

## **4.2 Results**

### **4.2.1 I229C Concentration Response Curve Data**

The I229C mutant was tested for its response to glycine in a concentration response curve. The glycine EC<sub>50</sub> values and the Hill slopes for this mutant were compared to the wild type receptor. The I229C was significantly more sensitive to the agonist, with a reduction in the EC<sub>50</sub> values (Table 2).

**Table 2**

Glycine EC<sub>50</sub> and Hill coefficients for the wild type (WT) receptor and I229C mutant.

The average glycine EC<sub>50</sub> and Hill coefficients were experimentally calculated from fits of concentration response curves from single oocytes and are expressed as a mean  $\pm$  S.E. of 6 to 7 oocytes.

Glycine Receptor	EC <sub>50</sub> ( $\mu$ M)	Hill Coefficient
WT	280 $\pm$ 47	2.3 $\pm$ 0.56
I229C	110 $\pm$ 7**	3.5 $\pm$ 0.48

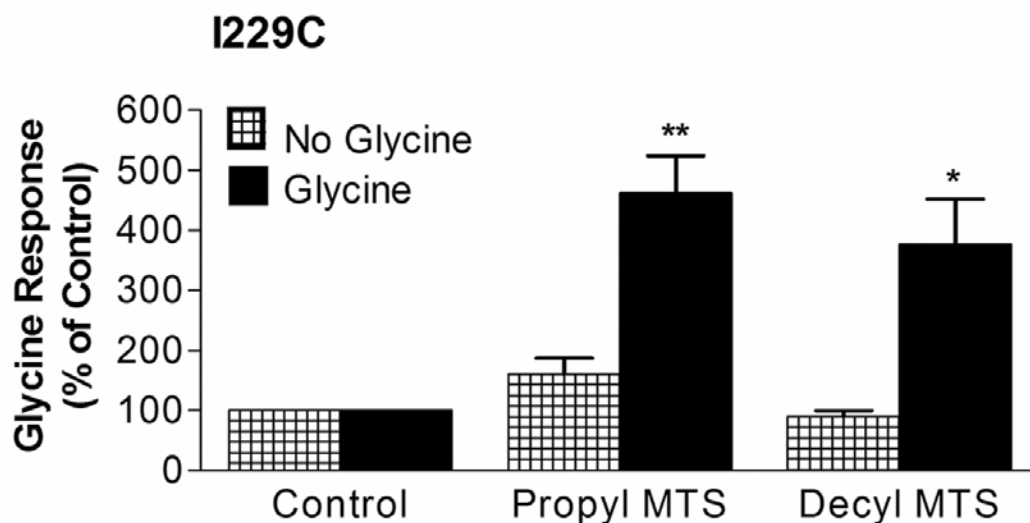
\*\*  $p < 0.01$ ; significantly different from WT receptor by the unpaired Student's  $t$ -test.



#### **4.2.2 Effects of MTS Reagents on I229C**

Accessibility of MTS reagents to I229 were tested in both the presence and absence of glycine. For I229C, propyl MTS (500  $\mu$ M) resulted in significant enhancement after application in the presence of glycine, but caused no change after application in the absence of glycine. Likewise, decyl MTS (50  $\mu$ M) showed labeling in the presence, but not in the absence of glycine (Figure 12).

These same MTS concentrations were tested for their effects on WT receptors. Control propyl MTS applications (500  $\mu$ M) on the WT did not produce an irreversible change in current after application in the absence ( $100 \pm 6$  % of control,  $n = 4$ ) or in the presence ( $110 \pm 4$  % of control,  $n = 4$ ) of 1 mM glycine. Control applications of decyl MTS (50  $\mu$ M) also did not change the WT receptor function in the absence ( $86 \pm 10$  % of control,  $n = 4$ ) or in the presence ( $83 \pm 6$  % of control,  $n = 4$ ) of 1 mM glycine.



**Figure 12. Covalent reaction of GlyR  $\alpha$ 1 mutant I229C with propyl and decyl MTS.** Propyl MTS (500  $\mu$ M) and decyl MTS (50  $\mu$ M) reacted and resulted in enhancement of receptor function only after application in the presence of 1 mM glycine. Data are expressed as a mean  $\pm$  S.E. of 5 to 7 oocytes. The paired Student's *t*-test was used to determine significance of differences in the glycine EC<sub>5-10</sub> before (control) and after treatment of MTS (\* *p* < 0.05 and \*\* *p* < 0.01).

### **4.2.3 I229C Responses to Alcohols and Volatile Anesthetics**

The responses of the I229C mutant to alcohols and anesthetic molecules was determined and compared to the WT receptor. Percent of control effects of ethanol (100 mM), octanol (115  $\mu$ M), isoflurane (0.8 mM) and chloroform (2.0 mM) were tested on an EC<sub>5-10</sub> concentration of glycine, which was determined for each oocyte.

The drug response profile of I229C was altered due to the substitution when compared to the WT responses. The two alcohols tested (ethanol and octanol) had no potentiating effect as seen in the WT receptor. The response to isoflurane was decreased significantly, while the response to chloroform was unchanged (Table 3).

**Table 3**

Responses of WT and I229C receptors to ethanol (100 mM), octanol (115  $\mu$ M), isoflurane (0.8 mM) and chloroform (2.0 mM). Percent of control responses were measured on the EC<sub>5-10</sub> glycine responses (determined for each oocyte).

Glycine Receptor	Glycine Response (Percent of Control)			
	Ethanol	Octanol	Isoflurane	Chloroform
WT	180 $\pm$ 11	270 $\pm$ 17	820 $\pm$ 82	300 $\pm$ 35
I229C	89 $\pm$ 8 ***	110 $\pm$ 8 ***	220 $\pm$ 21 ***	240 $\pm$ 58

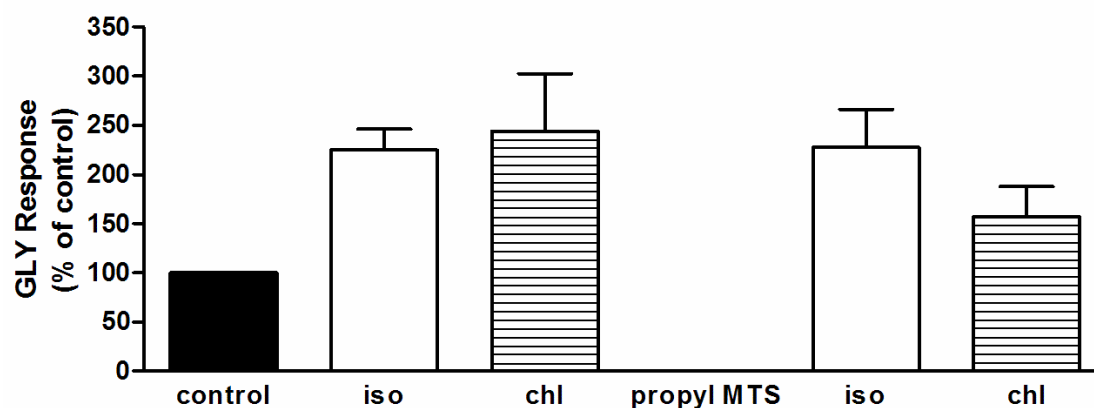
Data are expressed as a percent of control of the mean  $\pm$  S.E. of 4 to 18 oocytes.

Mutant responses were compared to the WT with the unpaired Student's *t*-test (\*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

#### **4.2.4 Effects of Volatile Anesthetics on I229C Before and After Reaction with Propyl MTS**

Since I229C reacts with MTS and is a water-accessible position, one can ask if the binding of MTS reagents at these positions could block further potentiation of the glycine receptor response by alcohols and anesthetics. This experimental procedure was previously used to test whether S267C was involved with drug binding or was an allosteric site affected by drugs (Mascia et al., 2000). Since MTS reaction blocked further drug effects at S267C, this indicated that the MTS reagents were permanently occupying the drug binding cavity and thereby preventing a other drug molecule from binding to and effecting the receptor.

The effects of isoflurane (0.8 mM) and chloroform (2.0 mM) were tested on the EC<sub>5-10</sub> glycine response of I229C. Propyl MTS was applied in the presence of 1 mM glycine for reaction with receptors, and the maximal glycine response and EC<sub>5-10</sub> glycine concentration was re-determined. Then the ability of propyl MTS (500  $\mu$ M) to alter the responses of these two volatile anesthetics was determined. Application of propyl MTS did not reduce or eliminate the volatile anesthetic potentiation in the I229C mutant (Figure 13).



**Figure 13. Effects of volatile anesthetics on I229C before and after application of propyl MTS in the presence of 1 mM glycine.** The potentiation of the glycine EC<sub>5-10</sub> current by isoflurane (iso; 0.8 mM) and chloroform (chl; 2.0 mM) was measured before and after the application of 500  $\mu$ M propyl MTS (applied in the presence of 1 mM glycine). Propyl MTS application produced no change in the responses of isoflurane and chloroform. Data are expressed as a mean  $\pm$  SEM of 3 to 8 oocytes. The unpaired Student's *t*-test was used to determine differences in the percent of control responses of isoflurane and chloroform before and after the propyl MTS application ( $p > 0.05$  for all comparisons).

### 4.3 Discussion

MTS reagents only reacted with I229C in the presence of glycine. Both propyl and decyl MTS showed state-dependent reaction, indicating a change in receptor conformation with channel gating allows I229C to react. This is an interesting finding since the major conformational changes due to channel gating occurring in the transmembrane domain of the receptor are usually considered to involve mainly the TM2 segment.

In comparing our results in the glycine receptor (Lobo et al., 2004a) with published data on TM1, there is little consistency in accessibility between the different subunits that have been examined (Table 4). The sequences were aligned using the consensus sequences of the ligand-gated ion channels by Bertaccini and Trudell (Bertaccini and Trudell, 2002). The only complete data sets on TM1 segments are from the mouse acetylcholine alpha subunit (Akabas and Karlin, 1995) and the mouse acetylcholine beta subunit (Zhang and Karlin, 1997). There have been no complete SCAM studies on any inhibitory ligand-gated ion channels to date. While I229C is reactive only in the presence of glycine, the aligned residue in the alpha subunit of the AChR (I220) is accessible only in the absence of neurotransmitter. Additionally, I220C reacts with the sulfhydryl-specific compound MTSEA, but does not react under either condition with MTSES. Meanwhile, in the AChR beta subunit, the aligned residue A231 is not reactive in the absence or presence of neurotransmitter with either MTSEA or MTSES. It seems that the positioning of this residue is quite variable between different subunits, which leads to these variable reactivity results (Table 4).

**Table 4**

TM 1 reactivity summary. Our reactivity data for I229C of the glycine receptor (Lobo et al., 2004a) is shown with other SCAM data published on TM1 from the acetylcholine receptor (Akabas and Karlin, 1995; Zhang and Karlin, 1997). The receptor subunit and sulfhydryl-specific reagents used in the experiments are indicated. The glycine receptor results were determined using an EC<sub>5-10</sub> test pulse of glycine. Akabas and Karlin and Zhang and Karlin both used a maximal test pulse of acetylcholine to determine changes in receptor function after reaction.



**Table 4 - TM1 Summary**

Position		Position				Position			
GlyRa1-human		AChR alpha-mouse				AChR beta-mouse			
	Lobo et al., 2004	Akabas and Karlin, 1995		Akabas and Karlin, 1995		Zhang and Karlin, 1997		Zhang and Karlin, 1997	
	propyl MTS	MTSEA		MTSES		MTSEA		MTSES	
	no gly	no ach		no ach		no ach		no ach	
	glycine	ach		ach		ach		ach	
L224		P211	↑	NR	NR	R219-P222	NR	NR	NR
L225		L212	NR	NR	NR	L223	NR	NR	NR
Q226		Y213	↑	NR	↑	F224	↑	↑	↑
M227		F214	NR	NR	NR	Y225	NR	↑	↑
Y228		I215	↑	NR	NR	L226	↓	NR	NR
I229		V216	↑	NR	NR	V227	NR	NR	NR
P230		N217	↑	NR	NR	N228	NR	NR	NR
S231		V218	NR	↓	NR	V229	NR	↑	NR
L232-W243	NR	I219	NR	NR	NR	I230	NR	NR	NR
		I220	↑	NR	NR	A231	NR	NR	NR
		P221				P232	NR	NR	NR
		C222 (WT)	NR	NR	NR	C233 (WT)	NR	NR	NR
		L223-F225	NR	NR	NR	I234-P247	NR	NR	NR

NR=no reaction

↑ increased response

↓ decreased response

In contrast to S267C (Mascia et al., 2000), while propyl MTS reaction occurred with I229C in the open state and caused an enhancement of receptor function, reaction with propyl MTS was unable to block further potentiation by the glycine receptor modulators isoflurane and chloroform. Reasons for this may be that the introduced MTS reagent is not the correct size or shape to mimic a drug molecule, and therefore prevent isoflurane and chloroform action. Testing a larger MTS reagent that would fill more of the putative drug binding cavity may resolve this question. Another possibility is that this position is located in the alcohol and volatile anesthetic binding cavity, but it is not playing a direct role in binding isoflurane and chloroform. However, since the I229C mutation eliminated the effect of ethanol and octanol and reduced the potentiation by isoflurane, the position does seem to be important for receptor potentiation by alcohols and anesthetics. Also, in other studies, mutations at this position have indicated that this position is important for drug action (Greenblatt and Meng, 1999; Jenkins et al., 2001). Currently, the mechanism for this action is not fully clear. Though, propyl MTS was unable to block potentiation by octanol and isoflurane, these two residues may play a role in stabilizing drug molecules in the binding cavity or allow a drug to be properly orientated to cause its effects.

## 5.0 Transmembrane Segment 2

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Lobo IA, Mascia MP, Trudell JR, Harris RA. Channel gating of the glycine receptor changes accessibility to residues implicated in receptor potentiation by alcohols and anesthetics. *J Biol Chem*. 2004 Aug 6;279(32):33919-27. Epub 2004 May 28.

### 5.1 Introduction to Transmembrane 2

An amino acid in TM2 (S267) of the glycine receptor is critical for the action of both alcohols and volatile anesthetics (Jenkins et al., 2001; Mihic et al., 1997; Ueno et al., 2000; Wick et al., 1998; Yamakura et al., 2001; Ye et al., 1998b). To study and identify water accessible residues of ion channels, such as those in drug binding pockets, methanethiosulfonate (MTS) reagents may be used as structural probes using the substituted cysteine accessibility method (Karlin and Akabas, 1998). MTS reagents rapidly react to form disulfide bonds with cysteines in the presence of water, and an irreversible change in receptor function is taken as evidence of disulfide bond formation.

By use of this method, residues accessible in the presence and/or absence of neurotransmitter to sulfhydryl-specific reagents have been determined for TM2 in GABA<sub>A</sub> and acetylcholine receptors (Horenstein et al., 2001; Xu and Akabas, 1996; Zhang and Karlin, 1998) and the TM2-TM3 loop for GABA<sub>A</sub> receptors (Bera et al., 2002). Lynch et al. demonstrated conformational changes occurring in the TM2-TM3 loop in the glycine receptor with gating (Lynch et al., 2001). Additionally, Mascia et al. (2000) found that covalent reaction of propyl methanethiosulfonate with a cysteine introduced in the putative alcohol/anesthetic binding site (S267C) of the glycine receptor irreversibly enhanced receptor function and abolished further potentiation by alcohols and anesthetics (Mascia et al., 2000).

Glycine receptors predominate in the spinal cord and brain stem and are present in the ventral tegmental area, a brain region of importance in the rewarding effects of alcohol (Betz, 1991; Eggers et al., 2000; Langosch, 1995; Legendre, 2001; Ye et al., 2001a). Clinically relevant concentrations of ethanol, longer chain alcohols and volatile anesthetics enhance the function of the glycine receptor (and the homologous GABA<sub>A</sub> receptor) in heterologous expression systems (Krasowski et al., 1998; Mascia et al., 1996a; Mascia et al., 1996b). Numerous studies have shown ethanol potentiation of glycine activated currents in cultured cells, including neurons of the hippocampus and ventral tegmental area, brain synaptoneurosomes, and mouse and chick spinal cord neurons (Aguayo and Pancetti, 1994; Aguayo et al., 1996; Celentano et al., 1988; Eggers et al., 2000; Engblom and Akerman, 1991; Ye et al., 2001a). As mediators of inhibition in the nervous system, glycine receptors may be involved in the sedative and anesthetic

effects of alcohol, a hypothesis supported by a recent study showing decreased alcohol effects in transgenic mice expressing a mutant, alcohol resistant,  $\alpha 1$  subunit (Findlay et al., 2002). The glycine receptor is one of the most credible candidates for mediating immobility caused by volatile anesthetics (Sonner et al., 2003).

These studies raise the question of the mechanism by which occupation of this protein cavity by alcohols, anesthetics or MTS reagents facilitates activation (or prevents inactivation) of the channel. It is established that channel gating causes tertiary structural rearrangements within receptor subunits (Spencer and Rees, 2002), so we were interested in how channel gating causes changes in accessibility to the alcohol and anesthetic binding pocket. We propose that the volume of this cavity, bounded by amino acids in TM1, TM2, and TM3, is larger in the open state of the channel than in the closed state. This would provide a mechanism by which occupation of the cavity by diverse small molecules can change receptor function. Our experiments expand on previous work (Bera et al., 2002; Horenstein et al., 2001; Lynch et al., 2001; Mascia et al., 2000; Williams and Akabas, 1999; Williams and Akabas, 2000; Williams and Akabas, 2001; Williams and Akabas, 2002; Xu and Akabas, 1996; Zhang and Karlin, 1998) to use alkyl MTS compounds of different lengths as molecular instruments to estimate the volume of the drug-binding pocket. In order to map the shape and organization of this binding cavity, we introduced cysteines at seven positions in TM2. We studied the ability of MTS reagents of different lengths to covalently react with these seven positions in both the open and closed conformations of the glycine receptor.

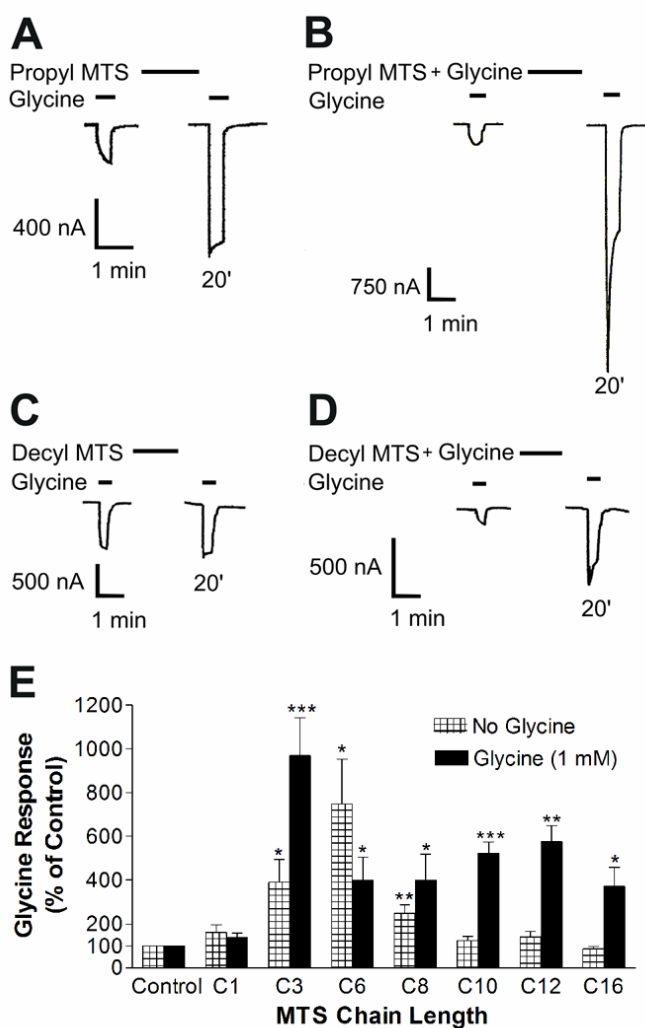
## 5.2 Results

### 5.2.1 MTS Reactivity at S267C and WT Controls

We first tested the ability of MTS reagents of different lengths to covalently react with a cysteine introduced at amino acid residue 267 (S267C). Propyl MTS irreversibly potentiated the glycine response after being applied in both the absence of glycine (Figure 14A) and in the presence of 1 mM glycine (Figure 14B). In contrast, decyl MTS failed to irreversibly potentiate the S267C response following application in the absence of glycine (Figure 14C), but could react and irreversibly enhance S267C when applied in the presence of glycine (Figure 14D).

We extended these observations by testing a series of MTS compounds of different sizes ranging from C1 (methyl) to C16 (hexadecyl) to determine their ability to react with S267C. These neutral MTS reagents have structural similarities to alcohols and anesthetics. Exposure to MTS reagents was carried out in the absence of glycine (closed state) and in the presence of a maximal concentration of glycine (1 mM, open and desensitized states). Methyl MTS did not cause receptor enhancement after application in either the closed or the open state. We found that MTS compounds of shorter chain lengths (propyl to octyl MTS) reacted with S267C when applied in both the presence and the absence of glycine, but the longer chain MTS compounds (decyl to hexadecyl MTS) were able to irreversibly react and alter the glycine response only when applied in the presence of glycine (Figure 14E). In all cases, except hexyl MTS, the enhancement observed was greater after the MTS reagent was applied in the presence of glycine, but

the enhancement values by hexyl MTS in the open and closed states were not significantly different.



**Figure 14. Effect of MTS reagents of different chain lengths on GlyR  $\alpha 1$ (S267C).** A) and B) The glycine current resulting from an EC<sub>5-10</sub> of glycine is enhanced 20 minutes after application of 50  $\mu$ M propyl MTS in the absence and presence of glycine (1 mM). C) The glycine current is not enhanced significantly when 50  $\mu$ M decyl MTS is applied in the absence of glycine. D) Significant enhancement of receptor function occurs after 50  $\mu$ M decyl MTS is co-applied with 1 mM glycine. E) MTS compounds (50  $\mu$ M) with chain lengths ranging from propyl (C3) to hexadecyl (C16) were found to irreversibly enhance the receptor when co-applied in the presence of glycine (1 mM). However, when applied in the absence of glycine, propyl through octyl (C8) MTS could cause a significant enhancement and longer MTS compounds had no effect on receptor function. Data are expressed as mean  $\pm$  S.E. of 6-13 oocytes. The paired Student's *t*-test was used to determine significance of differences in the glycine EC<sub>5-10</sub> responses before (control) and after treatment of MTS (\* *p* < 0.05, \*\* *p* < 0.01, and \*\*\* *p* < 0.001).



In addition to the n-alkyl MTS compounds, we tested a sulfhydryl-specific reagent of a different shape as well as a charged reagent. We observed that benzyl MTS and the negatively charged pCMBS<sup>-</sup> both caused enhancement of S267C after application in both conditions, but had no effect on the wild type (Table 5).

Wild type receptors did not show an irreversible change in function following application of any of the MTS compounds at 50  $\mu$ M (Table 5). Also, the highest concentrations of propyl and decyl MTS used in our studies had no effect on wild type glycine receptor function. Application of 1 mM propyl MTS (90 s) resulted in no significant change in current from control in either the absence ( $92 \pm 9$  % of control,  $n = 4$ ) or the presence ( $86 \pm 5$  % of control,  $n = 4$ ) of 1 mM glycine. Likewise, decyl MTS (300  $\mu$ M, 90 s) resulted in no significant change in either the absence ( $91 \pm 3$  % of control,  $n = 4$ ) or the presence ( $88 \pm 9$  % of control,  $n = 5$ ) of 1 mM glycine.

**Table 5**

Glycine responses, expressed as percent of control, of wild type and GlyR  $\alpha 1$ (S267C) receptors following application of sulfhydryl-specific reagents of different sizes.

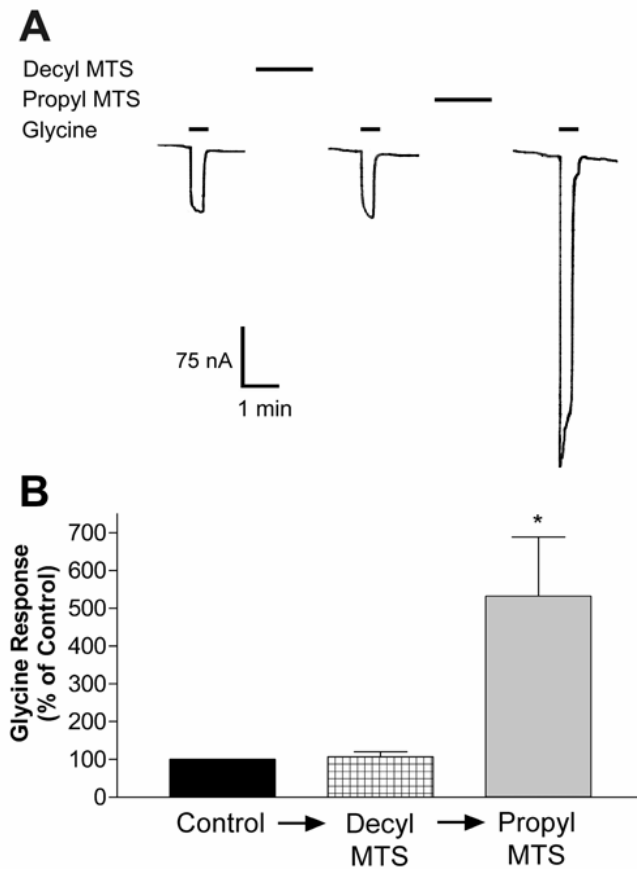
Glycine responses of receptors 20 minutes following a 50  $\mu$ M application of MTS compounds or pCMBS<sup>-</sup> in either the absence of glycine or in the presence of 1 mM glycine. Responses are expressed as percent of control initial EC<sub>5-10</sub> responses before MTS application, and represent a mean  $\pm$  S.E. of 4 to 13 oocytes.

MTS Chain Length	WT (No Gly)	WT (1 mM Gly)	S267C (No Gly)	S267C (1 mM Gly)
C1	100 $\pm$ 5	120 $\pm$ 9	160 $\pm$ 35	140 $\pm$ 22
C3	110 $\pm$ 11	92 $\pm$ 11	390 $\pm$ 100*	970 $\pm$ 170***
C6	87 $\pm$ 9	110 $\pm$ 13	750 $\pm$ 200*	400 $\pm$ 110*
C8	95 $\pm$ 5	88 $\pm$ 8	250 $\pm$ 39**	400 $\pm$ 120*
C10	86 $\pm$ 10	83 $\pm$ 6	120 $\pm$ 21	520 $\pm$ 53***
C12	110 $\pm$ 5	92 $\pm$ 7	140 $\pm$ 24	580 $\pm$ 74**
C16	88 $\pm$ 6	100 $\pm$ 7	86 $\pm$ 10	370 $\pm$ 85*
Ring-Substituted: Benzyl MTS	73 $\pm$ 13	85 $\pm$ 6	2200 $\pm$ 670*	1700 $\pm$ 340**
Charged: pCMBS <sup>-</sup>	82 $\pm$ 11	98 $\pm$ 12	500 $\pm$ 98**	510 $\pm$ 120*

$p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ; significantly different from control before MTS by Student's paired *t*-test.

### **5.2.2 Testing for Silent Reaction of Long Chain MTS Compounds at S267C**

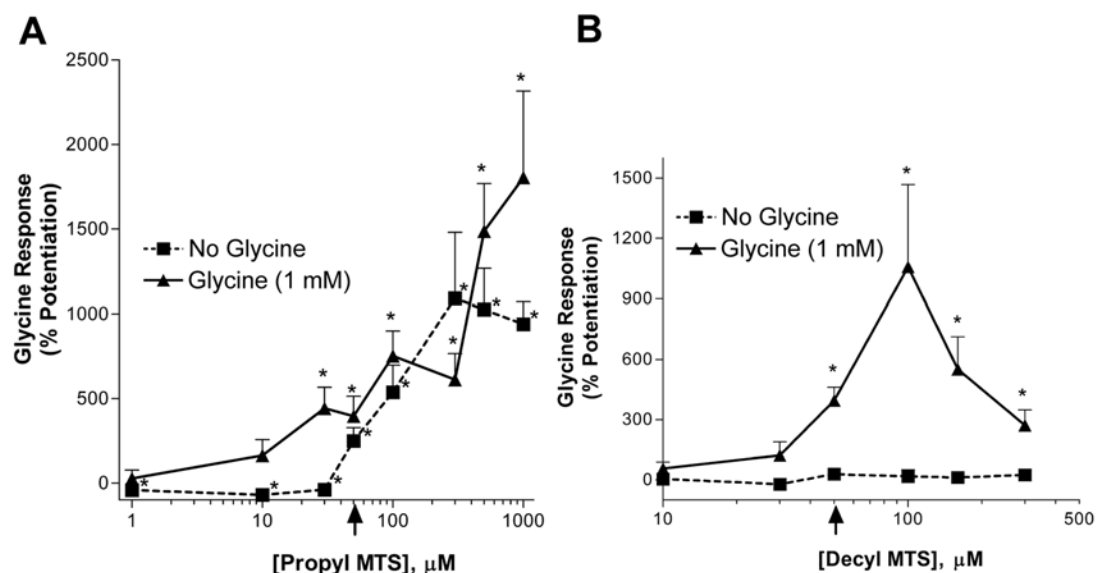
Of consideration was the possibility that although longer chain MTS compounds did not cause enhancement of the glycine response, they could be “silently” reacting with the receptor without altering receptor function. In this way, the presence of MTS would go undetected. This possibility was tested by exposing the receptor to 100  $\mu$ M decyl MTS (a compound having no effect following application in the closed state) and measuring the glycine response, and following this with a subsequent application of 50  $\mu$ M propyl MTS (a compound that caused significant potentiation following application in the closed state) and measuring the glycine response in the same oocyte (Figure 15). Decyl MTS produced no change in the glycine response, and the subsequent application of propyl MTS produced a percent enhancement of  $530 \pm 160$ , a value not statistically different from the  $390 \pm 100$  percent enhancement viewed following a single application of propyl MTS to the S267C mutant in the absence of glycine (Table 5).



**Figure 15. Decyl MTS does not block action of propyl MTS when applied in the closed state to GlyR  $\alpha 1$ (S267C).** To ensure that long chain MTS compounds were unable to label the cysteine in the closed state, labeling with 100  $\mu$ M decyl MTS (no significant effect) was followed by a subsequent application of 50  $\mu$ M propyl MTS (significant potentiation). A) This is an example tracing of the glycine responses and B) shows the mean  $\pm$  S.E. of the responses of 10 experiments. The  $EC_{5-10}$  was determined for each oocyte (average =  $48 \pm 8$   $\mu$ M). The paired Student's *t*-test was used to determine significance of differences in the glycine  $EC_{5-10}$  responses before and after treatment of propyl and decyl MTS (\*  $p < 0.05$ ).

### 5.2.3 MTS Concentration Response Curves at S267C

Various concentrations of propyl and decyl MTS were tested for reaction with S267C in the absence of glycine or in the presence of 1 mM glycine (Figure 16). The time of MTS application was held constant at 90 s. Propyl MTS caused enhancement after application in both the presence (30  $\mu$ M and higher) and in the absence (50  $\mu$ M and higher) of glycine. Lower concentrations of propyl MTS (1, 10 and 30  $\mu$ M) resulted in a small but significant inhibition when applied in the absence of 1 mM glycine. The reaction reaches a maximum at 1 mM propyl MTS for both curves, with no statistical difference between the 500  $\mu$ M and 1 mM points (Figure 16A). Decyl MTS did not change glycine receptor function, irrespective of the concentration tested when applied in the absence of glycine. When applied in the presence of glycine, concentrations of decyl MTS of 50  $\mu$ M and greater resulted in enhancement, with the greatest percent potentiation after application of 100  $\mu$ M decyl MTS. The mean potentiation after application of 300  $\mu$ M decyl MTS was not significantly different from that produced by 100  $\mu$ M, when compared by the Student's *t*-test (Figure 16B).



**Figure 16. Concentration response curves for propyl and decyl MTS in both the presence and absence of glycine for GlyR  $\alpha 1$ (S267C).** A) For propyl MTS, enhancement occurs when the propyl MTS is applied for 90 s in both the presence and in the absence of 1 mM glycine, increasing as the concentration is increased. Lower concentrations of propyl MTS (1, 10 and 30  $\mu\text{M}$ ) resulted in a small but significant inhibition only when applied in the absence of glycine. B) For decyl MTS, there was no change in glycine receptor function irrespective of the concentration applied (90 s) to the closed state of the receptor. When decyl MTS was applied in the presence of glycine, concentrations of 50  $\mu\text{M}$  and greater resulted in enhancement, with the greatest percent potentiation after application of 100  $\mu\text{M}$  decyl MTS. Arrows indicate the 50  $\mu\text{M}$  concentration used in the earlier S267C experiments. MTS reagents were applied for 90 s, and glycine applications were 30 s. Each concentration point represents 4-10 oocytes for the propyl MTS curve, and 4-11 oocytes for the decyl MTS curve. Data are expressed as a mean  $\pm$  S.E. The paired Student's *t*-test was used to determine significance of differences in the response to glycine EC<sub>5-10</sub> before and 10 minutes after treatment of propyl or decyl MTS (\* *p* < 0.05).

#### 5.2.4 Rates of Reaction with S267C

We examined the state dependence of propyl, hexyl and decyl MTS reaction with S267C by measuring the rate of reaction in the presence and absence of glycine (Table 6). (For decyl MTS, no reaction occurred in the absence of glycine, and the rate of reaction was only measured with glycine). The rate of reaction of propyl MTS in the presence of 1 mM glycine ( $\tau = 29 \pm 6.5$  s,  $k = 1050 \pm 190$  s<sup>-1</sup>M<sup>-1</sup>) was significantly faster than in the absence ( $\tau = 138 \pm 20$  s,  $k = 169 \pm 29$  s<sup>-1</sup>M<sup>-1</sup>). The rate of hexyl MTS reaction with S267C was also faster in the presence of glycine, and the reaction rates increased with increasing MTS chain lengths (Table 6).

**Table 6**

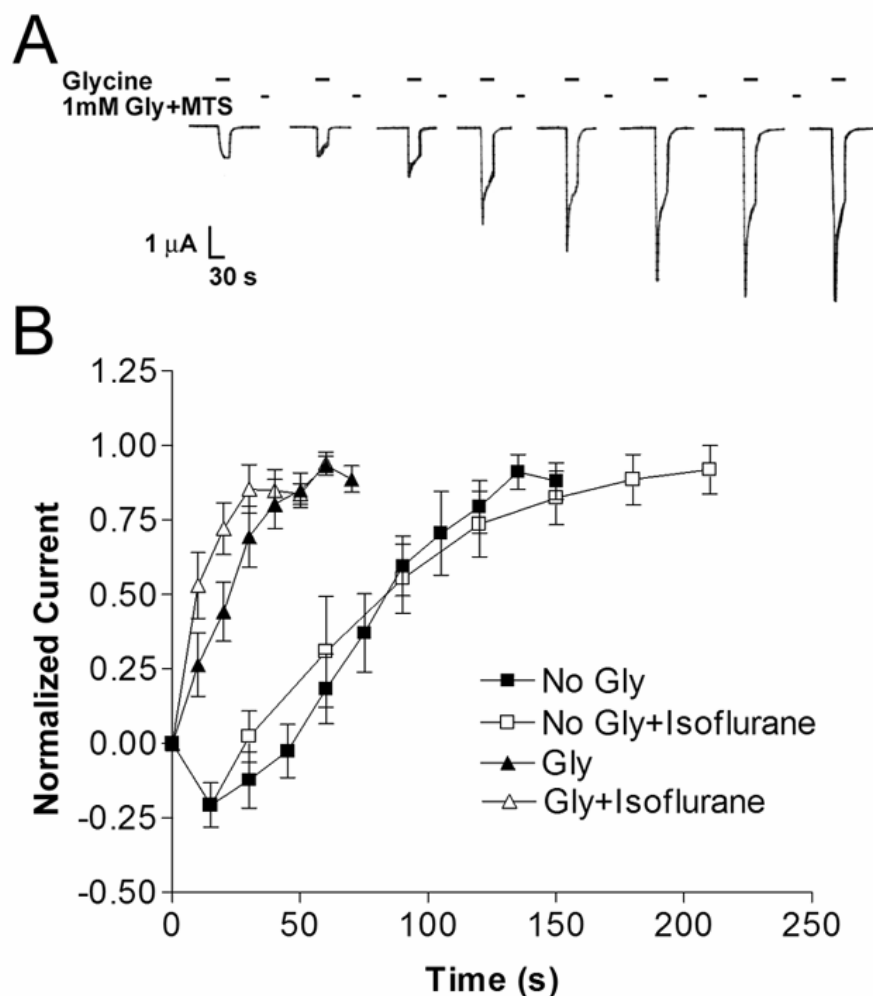
Rates of reactions of propyl, hexyl and decyl MTS with S267C in the absence of glycine and in the presence of 1 mM glycine. The steady-state rates of reaction of both propyl MTS and hexyl MTS increased significantly in the presence of glycine. Additionally, reaction rates increased with increasing MTS chain length. Rates are expressed as a mean  $\pm$  S.E. of 3 to 12 oocytes.

MTS Chain Length	No Glycine ( $\text{s}^{-1}\text{M}^{-1}$ )	1 mM Glycine ( $\text{s}^{-1}\text{M}^{-1}$ )
Propyl MTS	169 $\pm$ 29	1050 $\pm$ 190 **
Hexyl MTS	629 $\pm$ 91	10700 $\pm$ 1400 **
Decyl MTS	no reaction	70600 $\pm$ 7400

\*\*  $p < 0.01$ ; significantly different from no glycine rate of reaction by Student's  $t$ -test.



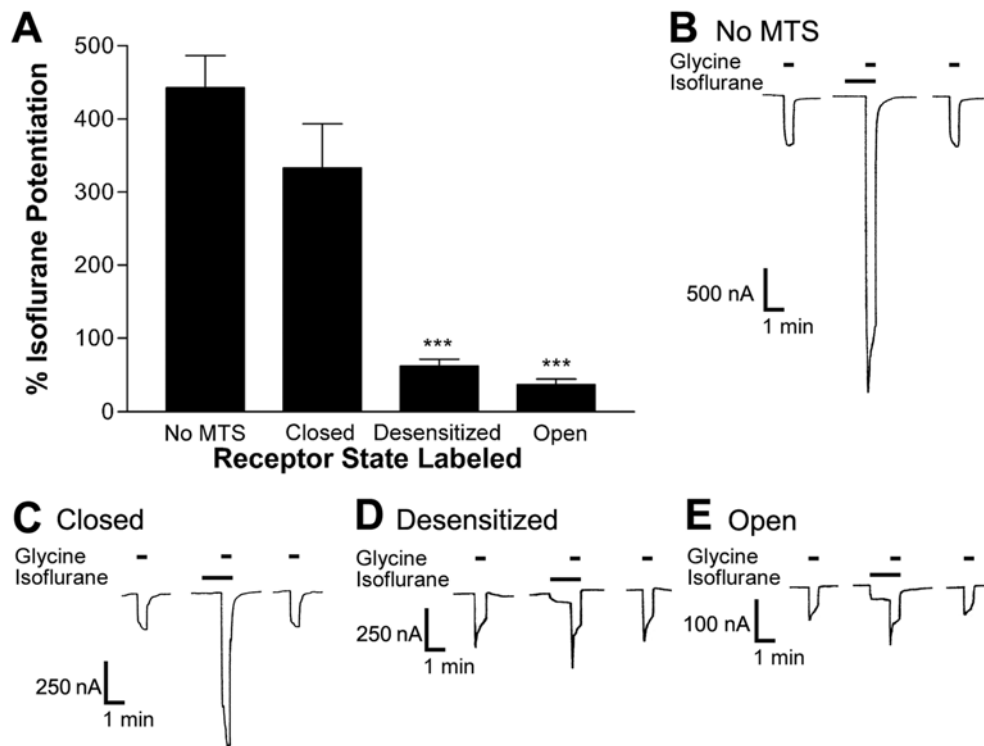
We also determined the rates of reaction of propyl MTS in both states in the presence of isoflurane, hypothesizing that the presence of an anesthetic could slow the reaction of MTS with S267C. The presence of isoflurane (0.6 mM) did not change the rate of propyl MTS reaction significantly in the absence ( $\tau = 124 \pm 41$  s,  $k = 220 \pm 41$  s<sup>-1</sup>M<sup>-1</sup>) or in the presence of 1 mM glycine ( $\tau = 19 \pm 4.5$  s,  $k = 1300 \pm 280$  s<sup>-1</sup>M<sup>-1</sup>) (Figure 17).



**Figure 17. Rate of reaction of propyl MTS with S267C in four conditions.** A) An example tracing of the reaction of S267C with 50  $\mu$ M propyl MTS is shown above. The effect of these short exposures on the EC<sub>5-10</sub> current of S267C was tested. Each short exposure was 15 seconds long and the initial EC<sub>5-10</sub> glycine (30 s) followed after 10 minutes of washout. This procedure was continued until the potentiation reached a stable plateau. B) The rates of reaction of 50  $\mu$ M propyl MTS with S267C were determined in four conditions: 1) in the absence of glycine, 2) 0.6 mM isoflurane in the absence of glycine, 3) in the presence of 1 mM glycine, 4) 0.6 mM isoflurane in the presence of glycine. The currents were normalized and graphed for comparison and the pseudo-second order rates were determined. Reaction was significantly faster in the presence of glycine in both pairs of conditions. Reactions were not altered significantly by the co-application of 0.6 mM isoflurane.

### 5.2.5 Which S267C Receptor States are Reactive?

One possible explanation of the MTS labeling results obtained in the presence of glycine is that MTS is covalently reacting with and stabilizing both the open and desensitized states of the receptor. To differentiate between these two states, potentiation by isoflurane (0.8 mM) was tested following labeling S267C receptors (with 50  $\mu$ M decyl MTS) in three states: closed (as a control), desensitized and open (Figure 18; see Materials and Methods). The potentiation values for each condition were compared to the isoflurane potentiation of unlabeled receptors with the hypothesis that receptors that reacted with MTS would have eliminated or reduced isoflurane potentiation, as previously demonstrated (Mascia et al., 2000). As expected, isoflurane potentiation following labeling in the closed state (Figure 18C) did not differ from that of unlabeled receptors (Figure 18B), further supporting the conclusion that decyl MTS is unable to react in the closed state. MTS appears to react with and stabilize both the desensitized (Figure 18D) and open states (Figure 18E). Following reaction of decyl MTS, isoflurane produced a current independent of glycine, as shown in the tracings, likely indicating that some channels were open in the absence of glycine.



**Figure 18. Isoflurane potentiation of the EC<sub>5-10</sub> glycine response of S267C following labeling of the receptor in different states with 50  $\mu$ M decyl MTS.** A) The potentiation of the EC<sub>5-10</sub> glycine response by 0.8 mM isoflurane was measured on unlabeled receptors (B, No MTS) as the control. Following application of 50  $\mu$ M decyl MTS in the absence of glycine (closed) or in the presence of 1 mM glycine (open), the potentiation by isoflurane was measured. Potentiation by isoflurane was also measured on receptors labeled in the “desensitized” state, where reaction with 50  $\mu$ M decyl MTS followed application of 1 mM glycine for 11-14 minutes to desensitize receptors. C) The closed state was not labeled by decyl MTS because there was no significant elimination in the isoflurane potentiation. Both the desensitized and open states were labeled by decyl MTS, resulting in significant reduction of isoflurane potentiation. In addition, isoflurane alone potentiated receptors after reaction with decyl MTS in the desensitized and open states indicating that these receptors now had tonic activity (D and E). Data are expressed as a mean  $\pm$  S.E. of 6 to 9 oocytes. The average current elicited by EC<sub>5-10</sub> of glycine before and after each isoflurane application was used to calculate the percent potentiation for each condition. The mean average currents (nA)  $\pm$  S.E. produced by an EC<sub>5-10</sub> of glycine are as follows: no MTS =  $550 \pm 100$ , closed =  $400 \pm 70$ , desensitized =  $330 \pm 120$ , and open =  $230 \pm 50$ ). One-way ANOVA with the Dunnett’s post test was used to determine significance of differences in the isoflurane potentiated glycine EC<sub>5-10</sub> of the labeled receptors versus the control, “No MTS” isoflurane response in unlabeled receptors (\*\*\*  $p < 0.001$ ).

### 5.2.6 Effect of Strychnine After Reaction of MTS with S267C

It has been shown that MTS reaction at a position homologous to GlyR  $\alpha 1$ (S267C) in the 5-HT<sub>3</sub> receptor (L293C) resulted in channels locked in the open state (Reeves et al., 2001). We tested for this possibility by applying strychnine (10  $\mu$ M) to S267C receptors following MTS labeling. There was no effect of strychnine after propyl MTS (50  $\mu$ M) was applied in either the open or the closed state ( $n = 4-6$ ). Predictably, there was also no effect of strychnine after application of 50  $\mu$ M decyl MTS to the oocytes in the closed state ( $n = 5$ ). However, following decyl MTS (50  $\mu$ M) reaction in the presence of 1 mM glycine, 10  $\mu$ M strychnine blocked a small inward current of  $110 \pm 34$  nA ( $n = 7$ ), indicating that some channels were constitutively open following labeling. In some cases, MTS reagents produced a current when applied in the absence of glycine on mutant receptors as seen in studies at this position in the GABA receptor with pCMBS<sup>-</sup> (Bali and Akabas, 2004). The currents observed here were blocked by 10  $\mu$ M strychnine indicating that MTS alone can open some channels. In all cases, the currents produced by 50  $\mu$ M MTS alone were very small (never exceeding 1 % of the maximal current), returned to baseline after the application and were never observed in the WT. Most often, we did not observe a current induced by MTS alone at all. Because the MTS alone current is not appreciable, this should not change our interpretation of data for reactions in the closed state.

### 5.2.7 MTS Volumes After Reaction at S267C

The volumes of MTS reagents before and after reaction were calculated using Spartan 5.0 (Wavefunction, San Diego, CA). The relevant volume for diffusion into the binding site is the whole molecular volume of the MTS reagent, whereas the relevant volume for functional analysis is the portion of the MTS molecule that reacts covalently with the substituted cysteine residue and causes the observed effects. For each functional MTS volume that has covalently reacted given below, the sulfinic acid leaving group contributes approximately  $65 \text{ \AA}^3$  to the MTS reagent volumes. Propyl MTS ( $102 \text{ \AA}^3$ ) through octyl MTS ( $204 \text{ \AA}^3$ ) were able to covalently react with both open and closed states of S267C to produce enhancement of the glycine response. Benzyl MTS, with a functional volume of  $150 \text{ \AA}^3$ , caused the largest responses after reaction in both states. Hexadecyl MTS ( $368 \text{ \AA}^3$ ) was the largest compound tested that could react in the presence of glycine to cause enhancement. Substituting cysteine ( $123 \text{ \AA}^3$ ) in place of serine ( $110 \text{ \AA}^3$ ) resulted in a volume increase of  $13 \text{ \AA}^3$ .

### 5.2.8 Glycine Concentration Response Curve Data for WT and TM2 Mutants

Additionally six other neighboring residues in TM2 were mutated to cysteine and tested for accessibility to MTS reagents to determine the “ceiling” and “floor” of the binding cavity. A recent nuclear magnetic resonance study of the TM2 segment (Tang et al., 2002), a consensus of ten secondary structure prediction algorithms for ligand gated-ion channels (Bertaccini and Trudell, 2002) and the cryo-electron micrograph structure of the acetylcholine receptor (Miyazawa et al., 2003) all provide evidence that TM2 is an

alpha helix. We selected the amino acids in TM2 that would be in close proximity to S267 in an alpha helix. The residues targeted by mutagenesis were M263, T264, V260, T259 and G256 (helical turns toward the cytoplasm from S267) and S270 (approximately a helical turn up toward the extracellular surface). The glycine EC<sub>50</sub> values and the Hill slopes for these mutants were compared to the wild type receptor (Table 7). The EC<sub>50</sub> values for T259C and A288C increased significantly from that of the wild type receptor. We chose propyl and decyl MTS to characterize these eight mutant receptors because of the labeling distinction we observed on S267C (Figure 14).

**Table 7**

Amino acid positions (Miller, 1989), glycine EC<sub>50</sub> and Hill coefficients for the wild type (WT) receptor and the cysteine substitution mutants studied. The glycine EC<sub>50</sub> and Hill coefficients were experimentally calculated from concentration response curves and are expressed as a mean  $\pm$  S.E. of 4 to 10 oocytes.

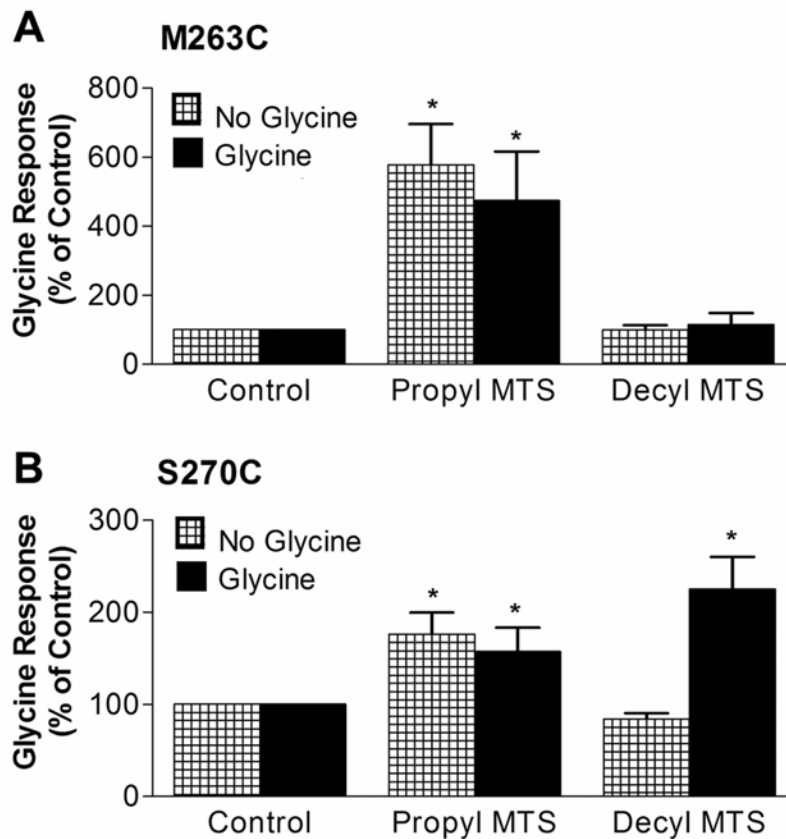
Glycine Receptor	TM2 Position	EC <sub>50</sub> ( $\mu$ M)	Hill Coefficient
WT	n/a	280 $\pm$ 47	2.3 $\pm$ 0.56
S270C	18'	370 $\pm$ 90	1.3 $\pm$ 0.18
S267C	15'	330 $\pm$ 56	1.1 $\pm$ 0.11
T264C	12'	69 $\pm$ 32	3.5 $\pm$ 2.4
M263C	11'	270 $\pm$ 80	3.0 $\pm$ 1.3
V260C	8'	41 $\pm$ 24	2.8 $\pm$ 1.1
T259C	7'	770 $\pm$ 110 **	1.8 $\pm$ 0.31
G256C	4'	670 $\pm$ 97 **	1.3 $\pm$ 0.11

\*\*  $p < 0.01$ ; significantly different from wild type receptors by one-way ANOVA with the Dunnett's post test.



### **5.2.9 Reaction of M263C and S270C with MTS Reagents**

For M263C, propyl MTS (500  $\mu$ M) enhanced receptor function when applied in both the presence and the absence of glycine. Decyl MTS (50  $\mu$ M) caused no enhancement of M263C in either condition (Figure 19A). For S270C, reaction with propyl MTS (500  $\mu$ M) caused an irreversible change in receptor function when applied in both the presence and the absence of glycine. Decyl MTS (50  $\mu$ M) was able to enhance S270C current after application in only the presence of glycine (Figure 19B).



**Figure 19. Labeling of GlyR  $\alpha$ 1 mutants M263C and S270C with propyl and decyl MTS.** A) GlyR  $\alpha$ 1(M263C): Propyl MTS (500  $\mu$ M) resulted in significant enhancement when applied in both the absence and presence of glycine (1 mM). Decyl MTS (50  $\mu$ M) produced no change in receptor function in either condition. B) GlyR  $\alpha$ 1(S270C): Propyl MTS (500  $\mu$ M) resulted in significant enhancement when the MTS was applied in both the absence and the presence of glycine (1 mM). Decyl MTS (50  $\mu$ M) only caused enhancement after being co-applied with 1 mM glycine. Data are expressed as a mean  $\pm$  S.E. of 4 to 8 oocytes. The paired Student's *t*-test was used to determine significance of differences in the glycine EC<sub>5-10</sub> before and after treatment of MTS (\* *p* < 0.05).

#### **5.2.10 Responses of WT, M263C and S270C to Ethanol, Octanol and Isoflurane**

For these two accessible mutants (M263C and S270C), the drug responses of ethanol, isoflurane and octanol were tested and compared to the WT receptor responses. The percent of control effects of ethanol (100 mM), by isoflurane (0.8 mM) and octanol (115  $\mu$ M) were tested on an EC<sub>5-10</sub> concentration of glycine, which was determined for each oocyte. The enhancing effect of ethanol seen in the WT was abolished by introducing the cysteine mutations at M263C and S270C. The effect of octanol was significantly reduced in both M263C and S270C. The potentiation by isoflurane was increased significantly by the S270C substitution (Table 8).

**Table 8**

Responses of WT, M263C and S270C receptors to ethanol (100 mM), octanol (115  $\mu$ M), and isoflurane (0.8 mM). Percent of control responses were measured on the EC<sub>5-10</sub> glycine responses (determined for each oocyte).

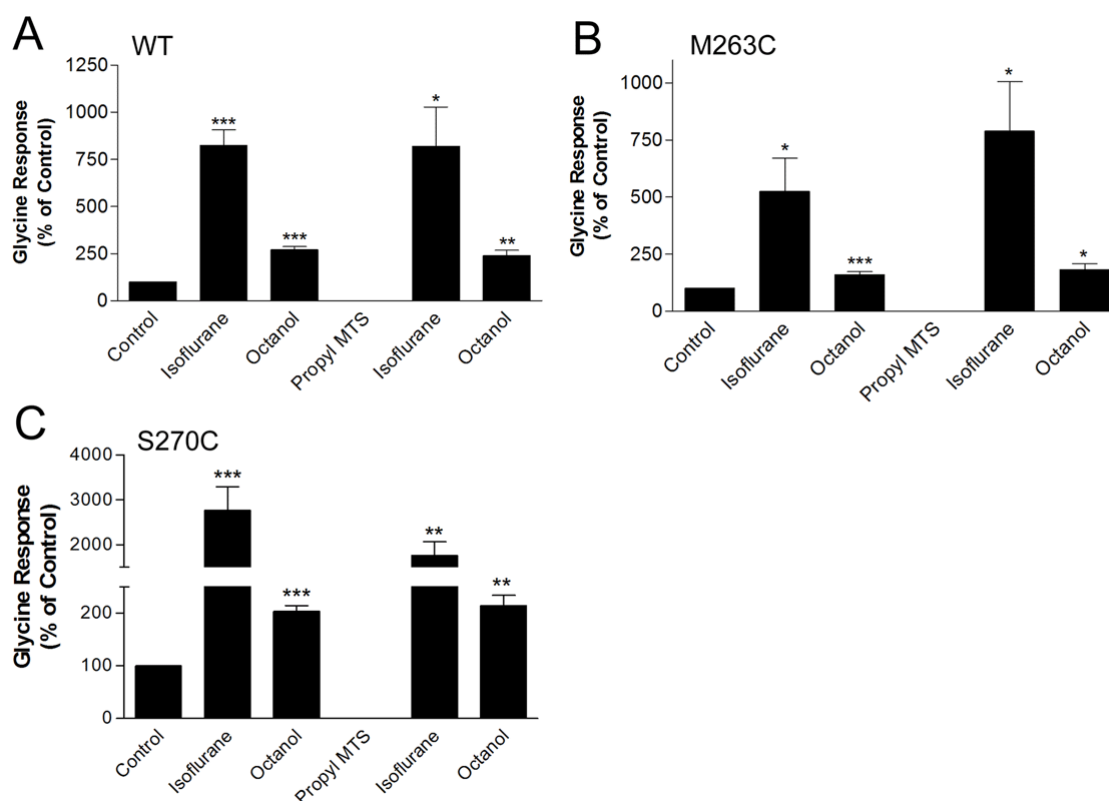
Glycine Receptor	Percent of Control		
	Ethanol	Octanol	Isoflurane
WT	180 $\pm$ 11	270 $\pm$ 17	820 $\pm$ 82
M263C	110 $\pm$ 10 **	160 $\pm$ 10 **	520 $\pm$ 150
S270C	120 $\pm$ 9 **	200 $\pm$ 11 **	2800 $\pm$ 530 **

Data are expressed as a percent of control of the mean  $\pm$  S.E. of 8 to 18 oocytes from 4-6 different frogs. Mutant responses were compared to the WT with the one-way ANOVA and the Dunnett's post-test (\*\*  $p < 0.01$ ).

### **5.2.11 Response of WT, M263C and S270C GlyRs to Octanol and Isoflurane Before and After Reaction with Propyl MTS**

Since M263C and S270C are reactive sites, a question that follows from this data is to ask whether the binding of MTS reagents at these positions could block further potentiation of the glycine receptor response by alcohols and anesthetics. This experimental procedure was used previously to test whether S267C was involved with drug binding or was an allosteric site affected by drugs (Mascia et al., 2000). MTS was able to block drug effects at S267C, providing evidence that the MTS reagents were occupying the drug binding cavity and preventing a drug molecule from causing its effects on the receptor.

The effects of octanol (115  $\mu$ M) and isoflurane (0.8 mM) were tested on the WT, M263C and S270C receptors. Then the ability of propyl MTS (500  $\mu$ M) to alter the responses of these two drugs was determined. Application of propyl MTS did not reduce or eliminate the alcohol or anesthetic potentiation of the WT or the M263C and S270C mutants (Figure 20).



**Figure 20. Effects of alcohols and anesthetics on GlyR $\alpha$ 1 (WT), GlyR $\alpha$ 1 (M263C) and GlyR $\alpha$ 1 (S270C) before and after application of propyl MTS.** The potentiation of the glycine EC<sub>5-10</sub> by isoflurane (0.8 mM) and octanol (115  $\mu$ M) were measured before and after the application of 500  $\mu$ M propyl MTS (in the presence of 1 mM glycine). A) Application of propyl MTS to the WT receptor produced no change in the response of the receptor to isoflurane and octanol. B) Application of propyl MTS to the M263C receptor produced no change in the response of the receptor to isoflurane and octanol. C) For S270C, propyl MTS application also produced no change in the response of the receptor to isoflurane and octanol. Data are expressed as a mean  $\pm$  SEM of 5 to 14 oocytes. The paired *t*-test was used to determine differences in the average glycine EC<sub>5-10</sub> response from before and after the drug application with the response during application of the drug (\* *p*<0.05, \*\* *p*<0.01, and \*\*\* *p*<0.001).

### **5.2.12 Inaccessible Mutants in TM2**

No labeling was observed for the other four mutants (G256C, T259C, V260C and T264C) under any condition tested: 500  $\mu$ M propyl MTS or 50  $\mu$ M decyl MTS in either the presence or absence of glycine (Table 9).

**Table 9**

Percent of control responses to EC<sub>5-10</sub> glycine following application of propyl or decyl MTS on wild type and TM2 mutant GlyRs in the presence or absence of glycine.

TM2 mutants (T264C, V260C, T259C, G256C), in proximity to S267, were tested for accessibility to MTS reagents. For the WT and T264C receptors, the maximal glycine concentration co-applied with propyl (500  $\mu$ M) and decyl MTS (50  $\mu$ M) was 1 mM, and for V260C, T259C and G256C, the maximal glycine concentration used was 10 mM.

Data are expressed as a mean  $\pm$  S.E. of 4 to 7 oocytes.  $p > 0.05$  for all receptor responses compared to the original EC<sub>5-10</sub> response (control) before MTS by the Student's paired *t*-test.

Glycine Receptor	Propyl MTS (No Gly)	Propyl MTS (Max Gly)	Decyl MTS (No Gly)	Decyl MTS (Max Gly)
WT	100 $\pm$ 6.4	110 $\pm$ 4.5	86 $\pm$ 10	83 $\pm$ 6.0
T264C	130 $\pm$ 33	85 $\pm$ 12	93 $\pm$ 24	139 $\pm$ 41
V260C	98 $\pm$ 6.5	88 $\pm$ 11	110 $\pm$ 9.0	110 $\pm$ 19
T259C	110 $\pm$ 6.6	103 $\pm$ 8.6	91 $\pm$ 13	91 $\pm$ 5.3
G256C	90 $\pm$ 3.7	103 $\pm$ 6.8	86 $\pm$ 6.8	80 $\pm$ 6.2



All of the results presented above were obtained by using an EC<sub>5-10</sub> concentration of glycine, determined individually for each oocyte. Mascia et al. previously determined that application of propyl MTS resulted in a leftward shift in the glycine concentration response curve with no change in the maximum glycine response (Mascia et al., 2000). Consistent with this, we found that current induced by 1 mM glycine in the S267C, M263C and S270C mutants was not significantly changed by exposure to propyl MTS.

### 5.3 Discussion

These results indicate that conformational changes occur in transmembrane segment two with channel gating. Experiments conducted on three mutants; M263C, S267C, and S270C, provide evidence that accessibility to the region of the putative alcohol and anesthetic binding pocket changes with channel gating. Under different conditions, MTS compounds covalently reacted at these positions to result in enhancement of glycine receptor function.

Experiments on S267C demonstrated that MTS reagents of longer lengths are able to react in the open state. Importantly, there is a distinct length (octyl MTS) after which larger MTS compounds do not react with substituted cysteines in the closed state, but do react in the open state. Methyl MTS, the smallest compound tested, did not cause a change in receptor function after application in either the closed or the open state, reinforcing the idea that a certain volume is needed to produce receptor enhancement. We noted the possibility that longer MTS compounds could “silently” react with the receptor without altering function, in which case we would not be able to detect the reaction. By showing that propyl MTS enhancement was not blocked by a previous application of decyl MTS, we determined that decyl MTS is unable to reach the cysteine in the closed state. Extrapolating, MTS compounds longer than decyl are not silently reacting with S267C and failing to enhance the receptor.

The concentration response curve for propyl MTS on S267C in the open and closed states indicated that higher concentrations caused greater enhancement than the 50  $\mu$ M concentration initially used (Mascia et al., 2000). The cause of inhibition with low

concentrations (or shorter applications) of propyl MTS and enhancement with higher concentrations (or longer applications) may represent differences in the number of glycine receptor subunits that have covalently reacted. Reaction of MTS with a single subunit may result in inhibition, while reaction with multiple subunits results in enhancement. Another possibility is that MTS may react when a cysteine is in two or more different conformational states.

The reaction rates of both propyl and hexyl MTS with S267C were faster in the presence of glycine than in the closed state, which provides further evidence of increased accessibility to S267 with channel opening. These rates of reaction with propyl MTS were not altered with the addition of isoflurane. It is interesting to note that propofol also did not protect the homologous TM2 positions from reaction with pCMBS<sup>-</sup> in a recent study in the GABA receptor  $\alpha 1$  and  $\beta 2$  subunit, while protection was only seen for the  $\beta 2$  subunit TM3 position (Bali and Akabas, 2004). This can be interpreted in at least two ways: 1) the on and off rates of anesthetic binding at S267 are too quick to provide observable competition with a compound that covalently reacts at its target or 2) the drug binding site is elsewhere and isoflurane is not competing with MTS to bind at S267C. Present data do not allow us to definitively distinguish between these two possibilities. We found that increasing the MTS chain length increased the rate of reaction. This may indicate that MTS compounds with longer chain lengths can reach the reactive cysteines more effectively through an amphipathic pathway or that the longer chain length MTS compounds are better stabilized near the reactive cysteine than shorter ones because of their lipophilic properties. It should be noted that the potency of *n*-alcohols for

potentiation of glycine receptor function increases with chain length (Mascia et al., 1996a; Wick et al., 1998).

When MTS reagents are coapplied with glycine, fractions of receptors exist in transitions between the closed, open and desensitized states. By testing isoflurane potentiation of S267C following decyl MTS application in different receptor states, we found that both the desensitized and open states could be labeled, as indicated by the elimination of the isoflurane potentiation. Additionally, a fraction of receptors were constitutively open following reaction with decyl MTS, suggesting that the open state had reacted and was stabilized.

For the three substituted amino acids that reacted (M263C, S267C, and S270C), we observe distinctions in which compounds are able to access the site. Some positions in the putative alcohol/anesthetic pocket accommodate longer MTS reagents in the presence of glycine, and others require agonist for reaction. We attribute this change in accessibility to a change in the size and shape of the drug binding cavity. An alternate hypothesis is that the access pathway to the cysteine has changed, allowing larger MTS reagents access to this region; however, such a mechanism does not appear to be sufficient to fully explain our data. In particular, if glycine only increased the size of an access pathway to the drug binding region, we would expect M263C to react with decyl MTS in the open state in the same manner as S267C and S270C. Since M263C reacts with propyl MTS, it must be in a water-filled cavity accessible to small MTS reagents. However, decyl MTS does not react with M263C, so access is not increased, and our data

is explained more accurately by a change in the volume/size of the drug binding cavity with channel gating.

In contrast to S267C (Mascia et al., 2000), though binding by MTS reagents was possible on mutants M263C and S270C and caused an enhancement of receptor function, reaction with propyl MTS was unable to block further potentiation of the glycine receptor modulators isoflurane and octanol. This may mean that the introduced MTS reagent is not the correct size or shape to mimic a drug molecule, and therefore prevent octanol and isoflurane action. Another possibility is that these two positions are located in the alcohol and volatile anesthetic binding cavity, but they are not playing a direct role in binding isoflurane. Still, both the M263C and S270C single mutations altered drug effects. Both eliminated the effect of ethanol, and M263C reduced the potentiation by isoflurane. Additionally, the potentiation by isoflurane was increased dramatically in the S270C mutant. Though, propyl MTS was unable to block potentiation the effects of octanol and isoflurane, these two residues may play a role in stabilizing drug molecules in the binding cavity or allow the drug to be properly orientated to cause its effects.

Mutations further toward the cytoplasm (G256C, T259C, V260C and T264C) were inaccessible to labeling by propyl and decyl MTS in the presence and absence of glycine. Reaction with MTS is much faster when the cysteine is in a water-filled environment (Karlin and Akabas, 1998) and our results suggest that if the water-filled cavity does extend to this depth, it is not of sufficient size to admit MTS reagents.

We compared our accessibility data for the glycine receptor (Lobo et al., 2004a) with the known reactivity data for TM2 in other ligand-gated ion channels (Akabas et al.,

1994; Goren et al., 2004; Panicker et al., 2002; Reeves et al., 2001; Williams and Akabas, 1999; Xu and Akabas, 1996; Zhang and Karlin, 1998) (Table 10). Of all of the TM segments, TM2 has been studied in the most detail, in the most subunits and with the most sulfhydryl-reactive reagents. In comparing the published SCAM data sets, there are many apparent differences and few commonalities. For comparison, the amino acid prime number positions (Miller, 1989) and the glycine receptor position will be used and sequences were aligned using the consensus sequences for ligand-gated ion channels (Bertaccini and Trudell, 2002). The 4' position (G256) was inaccessible in all receptors tested. The 7' and 8' positions were inaccessible in the glycine receptor, and most commonly inaccessible in the other receptors. While the 11' position (M263) was reactive in the glycine receptor, it was not accessible in any other receptor tested. The 15' position (S267) was accessible in both the glycine receptor and the GABA receptor subunits tested (Goren et al., 2004; Williams and Akabas, 1999; Xu and Akabas, 1996). In glycine receptors reaction at S267C resulted in potentiation, while reaction in the GABA receptor subunits tested always resulted in inhibition. The 15' position was not accessible in the AChR alpha subunit but reacted in the beta subunit (Akabas et al., 1994; Zhang and Karlin, 1998), and there were mixed results for this position in the 5-HT<sub>3</sub> receptor (Panicker et al., 2002; Reeves et al., 2001). The 18' position (S270) reacted in the GABA receptor beta 1 subunit (Goren et al., 2004) and in the 5-HT<sub>3</sub> receptor only with MTSEA (Panicker et al., 2002). In the other receptors tested, or with other compounds, this position was not reactive. The reactive residues differ from receptor to receptor and subunit to subunit making an overall amalgamation of these data sets

difficult in TM2. These differences in accessibility may play a role in the way each of these channels is gated. Since TM2 lines the channel pore, is responsible for gating, pore diameter and ion charge selectivity, variations in this segment of the protein may account for these functional differences.

**Table 10**

TM 2 reactivity summary. Our SCAM data for the TM2 glycine receptor mutants (Lobo et al., 2004a) is shown with other the known reactivity data for TM2 in other ligand-gated ion channels (Akabas et al., 1994; Goren et al., 2004; Panicker et al., 2002; Reeves et al., 2001; Williams and Akabas, 1999; Xu and Akabas, 1996; Zhang and Karlin, 1998).



**Table 10 - TM2 Summary**

TM2 Position	Position GlyRa1		Position GABA alpha1-rat		Position GABA beta1-rat	
		Lobo et al., 2004 propyl MTS no gly	glycine	Xu and Akabas, 1996; Williams and Akabas, 1999, pCMBS- no GABA	GABA	GABA
0'				NR	NR	
1'	R252			S251	NR	
2'	V253			V252	NR	
3'	G254			P253	NR	
4'	L255			E250-A254	NR	
5'	G256	NR	NR	R255	NR	
6'	I257			T256	NR	
7'	T258			V257	↓	A252
8'	T259	NR	NR	F258	NR	L253
9'	V260	NR	NR	G259	NR	G254
10'	L261			V260	NR	I255
11'	T262			T261	↓	T256
12'	M263	↑	↑	T262	NR	T257
13'	T264	NR	NR	V263	NR	V258
14'	T265			L264	↓	L259
15'	Q266			T265	↓	T260
16'	S267	↑	↑	M266	NR	M261
17'	S268			T267	NR	T262
18'	G269			T268	↓	T263
19'	S270	↑	↑	L269	NR	I264
20'	R271			S270	↓	S265
				I271	↓	T266
				S272	↓	H267
				A273	NR	L268
				R274	NR	R269
				N275	↑	E270

NR=no reaction

↑ increased response

↓ decreased response

**Table 10 - TM2 Summary (continued)**

TM2	Position	Position		Position		Position		Position	
Position	AchR alpha-mouse	AchR beta-mouse		5-HT3R		Panicker et al., 2002		Panicker et al., 2002	
	Akabas et al., 1994 MTSEA, MTSET, MTSES	Zhang and Karlin, 1998 MTSEA, MTSET, MTSES		Reeves et al., 2001 MTSET, MTSES		Panicker et al., 2002 MTSET		Panicker et al., 2002 MTSEA	
	no ach	ach	no ach	no 5-HT	5-HT	no 5-HT	5-HT	no 5-HT	5-HT
0'									
1'	↓	↓	NR	↑	NR	NR	NR	NR	NR
2'	no current	no current	NR	NR	NR	NR	NR	NR	NR
3'	NR	NR	NR	NR	NR	NR	NR	NR	NR
4'	↓	↓	NR	NR	NR	NR	NR	NR	NR
5'	NR	NR	NR	NR	NR	NR	NR	NR	NR
6'	↓	↓	NR	NR	NR	NR	NR	NR	NR
7'	NR	NR	NR	NR	NR	NR	NR	NR	NR
8'	↓	↓	NR	NR	NR	NR	NR	NR	NR
9'	↓	↓	NR	NR	NR	NR	NR	NR	NR
10'	↓	↓	NR	NR	NR	NR	NR	NR	NR
11'	NR	NR	NR	NR	NR	NR	NR	NR	NR
12'	NR	NR	NR	NR	NR	NR	NR	NR	NR
13'	↓	↓	NR	NR	NR	NR	NR	NR	NR
14'	NR	NR	NR	NR	NR	NR	NR	NR	NR
15'	NR	NR	NR	NR	NR	NR	NR	NR	NR
16'	↓	↓	NR	NR	NR	NR	NR	NR	NR
17'	NR	NR	NR	NR	NR	NR	NR	NR	NR
18'	NR	NR	NR	NR	NR	NR	NR	NR	NR
19'	NR	NR	NR	NR	NR	NR	NR	NR	NR
20'	↓	↓	NR	NR	NR	NR	NR	NR	NR

NR=no reaction

↑ increased response

↓ decreased response

Our data that the water-filled cavity between the TM segments does not extend further towards the cytoplasm concurs with other evidence in the GABA<sub>A</sub> receptor  $\beta$ 1 subunit that the cytoplasmic end of TM2 is tightly packed against the rest of the protein, while the extracellular half of the helix is more loosely packed (Goren et al., 2004). Thus, the putative alcohol and anesthetic binding pocket of the glycine receptor extends three alpha helical turns into the transmembrane segment from the extracellular surface. MTS reagents can enter from the extracellular side, but being blocked from diffusing or reacting at positions below M263. MTS reagents may enter via either a water-filled pathway or the lipid bilayer or a combination of the two, because both charged and neutral MTS compounds reacted. This drug binding region was suggested to correspond to the space between the five TM2 alpha helices and the ring of 15 alpha helices surrounding them in the recent crystallographic structure of the acetylcholine receptor (Miyazawa et al., 2003). Additionally, our data is consistent with the NMR structure of glycine receptor TM2 segments (Tang et al., 2002). In comparing the NMR structure of wild type and S267Y (anesthetic resistant) TM2 segments of the glycine receptor, Tang et al. noted that the mutant caused only local conformation changes. They predicted M263 could border the amphipathic drug binding cavity (Tang et al., 2002), which is supported by our MTS labeling data.

Previous work estimates the anesthetic binding site in the anesthetic-sensitive protein firefly luciferase to be 250 ml/mol (or 415 Å<sup>3</sup>/molecule of protein) (Franks and Lieb, 1984). Using a combination of mutagenesis and anesthetics of different sizes, Jenkins et al. estimated the volume of the anesthetic binding site in GABA<sub>A</sub> receptors to

be between 250 and 370 Å<sup>3</sup> (Jenkins et al., 2001). In our experiments, octyl MTS (269 Å<sup>3</sup>; 204 Å<sup>3</sup>, after reaction with the cysteine) was the largest compound to produce glycine receptor enhancement in the S267C mutant in both states. The largest compound tested, hexadecyl MTS, which affected the receptor only after application to the open state, has a molecular volume of 433 Å<sup>3</sup> (368 Å<sup>3</sup>, after reaction). This suggests that the volume of this cavity in the glycine receptor is similar to the anesthetic binding cavity in firefly luciferase and the GABA<sub>A</sub> receptor.

## 6.0 Transmembrane Segment 3

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Lobo IA, Mascia MP, Trudell JR, Harris RA. Channel gating of the glycine receptor changes accessibility to residues implicated in receptor potentiation by alcohols and anesthetics. *J Biol Chem*. 2004 Aug 6;279(32):33919-27. Epub 2004 May 28.

### 6.1 Introduction to Transmembrane Segment 3

An amino acid in TM3, A288, was identified a critical mediator of the potentiating effects of alcohols and/or volatile anesthetics on glycine receptors (Mihic et al., 1997). Extensive mutagenesis at this position showed that the molecular volume of the amino acids substituted for A288 were negatively correlated with volatile anesthetic action (Yamakura et al., 1999). This suggested that the volume of the putative volatile anesthetic and alcohol drug binding cavity was regulated by the size of the amino acid at the 288 position in TM3 (Yamakura et al., 1999). A288 is hypothesized to line an

alcohol and anesthetic binding pocket at the center of the four transmembrane segments of each receptor subunit (Yamakura et al., 2001).

These data make A288 an attractive target for mutagenesis and probing with MTS reagents using the substituted cysteine accessibility method. By use of this method, residues accessible in the presence and/or absence of neurotransmitter to sulfhydryl-specific reagents have been determined for TM3 in GABA<sub>A</sub> (Williams and Akabas, 1999) and the TM2-TM3 loop for GABA<sub>A</sub> receptors (Bera et al., 2002). Lynch et al. (2001) demonstrated conformational changes occurring in the TM2-TM3 loop in the glycine receptor with gating (Lynch et al., 2001). Additionally, Williams and Akabas have demonstrated that different GABA<sub>A</sub> receptor conformations are stabilized by the drugs diazepam and propofol using TM3 cysteine mutants (Williams and Akabas, 2000; Williams and Akabas, 2001; Williams and Akabas, 2002).

As it is known that conformational changes occur in TM3 of the GABA receptor with channel gating as well as the TM2-TM3 loop, we tested whether changes in accessibility occurred at A288 in the glycine receptor. In Chapter 4, we examined the ability of several differently sized, uncharged, MTS compounds to irreversibly occupy the S267C site of the glycine receptor under conditions where the channel was either closed (in the absence of glycine) or open (in the presence of glycine). From these results, it followed logically to extend these studies to A288C. Additionally, the rate of reaction of propyl MTS at A288C was determined and compared to the rate constants determined for S267C.

Previously, Mascia et al. demonstrated that covalent reaction of a drug analog (MTS) to S267C resulted in irreversible enhancement of receptor function and that the usual ability of octanol and isoflurane to enhance the receptor function was lost in the covalently bound receptors (Mascia et al., 2000). This provided evidence that the action of alcohols and anesthetics at this position was due to binding in this region and not because S267 was an allosteric site. In the present study, we examined this TM3 position for drug responses to ethanol, octanol and isoflurane. Additionally, the ability of sulfhydryl-specific methanethiosulfonate (MTS) anesthetic analog to covalently react with each of these positions and block alcohol and volatile anesthetic action was determined.

## **6.2 Results**

### **6.2.1 Concentration Response Curve Data**

The glycine EC<sub>50</sub> values and the Hill slopes for this mutant were compared to the wild type receptor (Table 11). The EC<sub>50</sub> values for A288C increased significantly from that of the wild type receptor.



**Table 11**

Glycine EC<sub>50</sub> and Hill coefficients for the wild type (WT) receptor and the cysteine substitution mutants studied. The glycine EC<sub>50</sub> and Hill coefficients were experimentally calculated from concentration response curves and are expressed as a mean  $\pm$  S.E. of 6 oocytes for each receptor type.

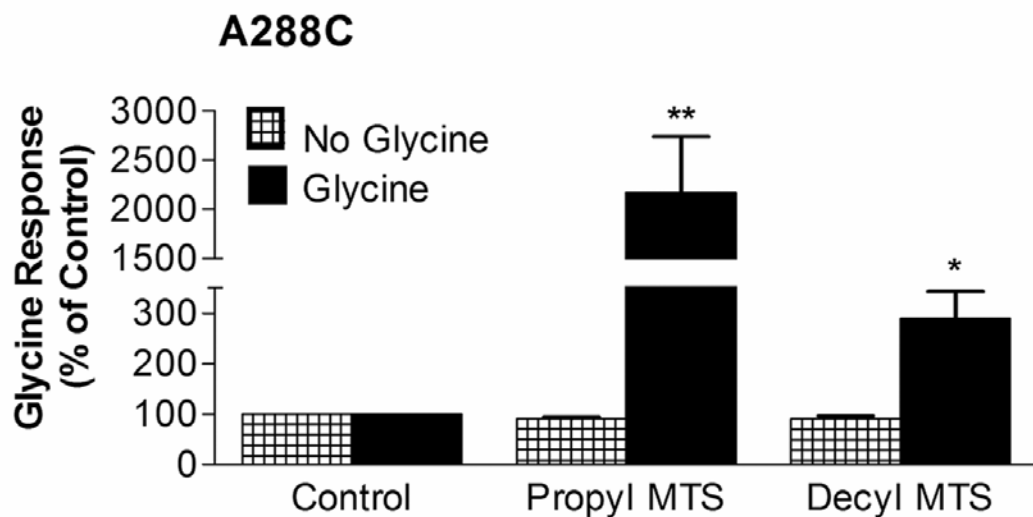
Glycine Receptor	EC <sub>50</sub> ( $\mu$ M)	Hill Coefficient
WT	280 $\pm$ 47	2.3 $\pm$ 0.56
A288C	1800 $\pm$ 270 **	2.0 $\pm$ 0.34

\*\*\*  $p < 0.001$ ; significantly different from WT receptor by the unpaired Student's  $t$ -test.

### 6.2.2 MTS Reactivity at A288C and WT Controls

We chose propyl and decyl MTS to characterize A288C for accessibility to this position in TM3 in the presence and absence of glycine, and tested the same concentrations on the WT receptor. Control propyl MTS applications (500  $\mu$ M) on the WT receptor had no effect in the absence ( $100 \pm 6$  % of control,  $n = 4$ ) or in the presence ( $110 \pm 4$  % of control,  $n = 4$ ) of 1 mM glycine. The control applications of decyl MTS (50  $\mu$ M) also did not change the WT receptor function in the absence ( $86 \pm 10$  % of control,  $n = 4$ ) or in the presence ( $83 \pm 6$  % of control,  $n = 4$ ) of 1 mM glycine.

In contrast, A288C showed state-dependence of accessibility. For A288C, a 500  $\mu$ M propyl MTS application resulted in significant labeling in the presence of 1 mM glycine, but no change after application in the absence of glycine (Figure 21). Decyl MTS (50  $\mu$ M) also showed labeling of A288C in the presence, but not in the absence of glycine (Figure 21). A lower concentration of propyl MTS (50  $\mu$ M) had no significant effect on the A288C receptor when applied in either the open or closed state (data not shown).

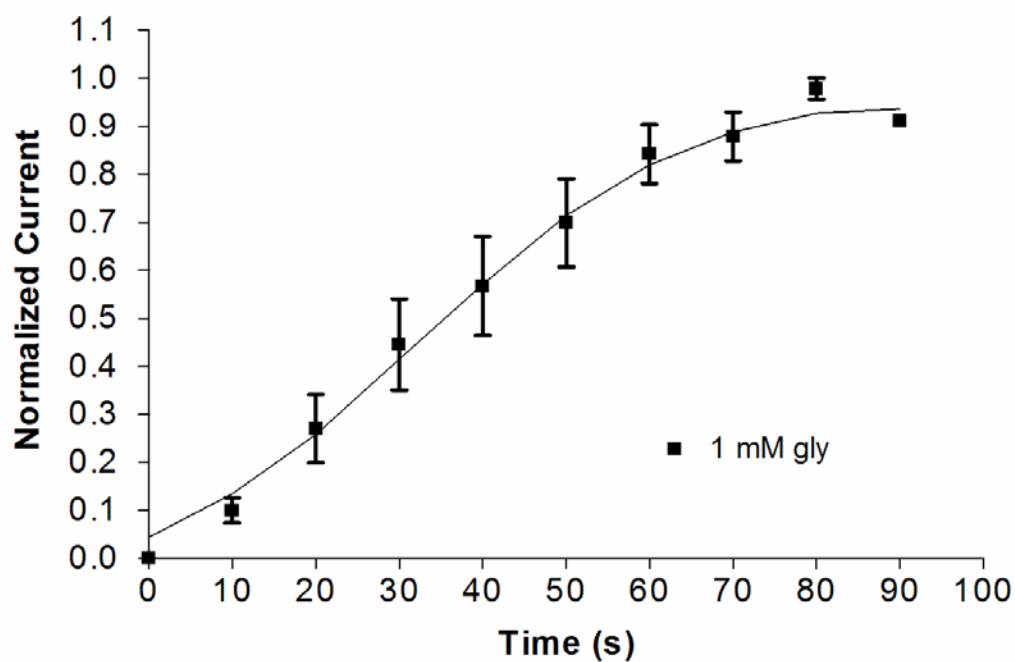


**Figure 21. Labeling of GlyR  $\alpha 1$  mutants A288C with propyl and decyl MTS.**

Propyl MTS (500  $\mu$ M) and decyl MTS (50  $\mu$ M) caused irreversible A288C receptor enhancement only when applied in the presence of 1 mM glycine (note that the scale of the Y axis is broken). Data are expressed as a mean  $\pm$  S.E. of 4 to 8 oocytes. The paired Student's *t*-test was used to determine significance of differences in the glycine EC<sub>5-10</sub> before and after treatment of MTS (\* *p* < 0.05 and \*\* *p* < 0.01).

### **6.2.3 Rates of Reaction of A288C with Propyl MTS**

The rate of reaction of propyl MTS (500  $\mu$ M) in the presence of glycine (1 mM) was measured for the A288C mutant. Because this reaction was state dependent (i.e. required glycine), the rate could not be measured in the absence of glycine. Ten second exposures of propyl MTS and glycine were applied until the reaction reached saturation point, or plateau, with no further increases in current with further applications. The mean curve of the normalized currents indicates a complete reaction after 90 s of cumulative exposure (Figure 22).



**Figure 22. Rate of reaction of propyl MTS with GlyR  $\alpha 1$ (A288C) in the presence of glycine.** Ten second exposures to 500  $\mu$ M propyl MTS and 1 mM glycine were co-applied to the A288C receptor until no further potentiation due to covalent reaction was measured. The currents from each oocyte were normalized and plotted against the cumulative length of propyl MTS exposure. The reaction was reached a plateau and was complete by 90 s.

#### **6.2.4 Comparison of TM3 Rate with TM2 Rate**

The rate constants for the reaction of propyl MTS with A288C were calculated and compared to the TM2 (S267C) reaction rate data from Chapter 5. While the TM2 site is accessible in both conditions, the TM3 position is only accessible in the presence of glycine. Additionally the reaction rate of S267C is significantly faster than that of A288C in the presence of glycine (Table 12).

**Table 12**

Rates of reactions of propyl MTS with S267C and A288C in the absence of glycine and in the presence of 1 mM glycine. The steady-state rates of reaction of propyl MTS is significantly faster with the TM2 site (S267C) than with TM3 site (A288C). Rates are expressed as a mean  $\pm$  S.E. of 6 to 12 oocytes.

Mutant	No Glycine ( $\text{s}^{-1}\text{M}^{-1}$ )	1 mM Glycine ( $\text{s}^{-1}\text{M}^{-1}$ )
S267C	169 $\pm$ 29	1050 $\pm$ 190
A288C	no reaction	52 $\pm$ 8.3 **

\*\*  $p < 0.01$ ; significantly different from the corresponding S267C rate of reaction by the unpaired Student's  $t$ -test.

### **6.2.5 Responses of A288C to Alcohols and Volatile Anesthetics**

The responses of A288C to alcohols and anesthetics were determined and compared to the WT receptor. Percent of control effects of ethanol (100 mM), octanol (115  $\mu$ M), and isoflurane (0.8 mM) were tested on an EC<sub>5-10</sub> of glycine. The glycine EC<sub>5-10</sub> was determined for each oocyte. The potentiation of ethanol and octanol was abolished in the A288C mutant. The effect of isoflurane was significantly reduced in A288C compared to the WT glycine receptor (Table 13).



**Table 13**

Percent of control responses of WT and A288C receptors to ethanol (100 mM), octanol (115  $\mu$ M), isoflurane (0.8 mM) and chloroform (2.0 mM). Percent of control responses were measured on the EC<sub>5-10</sub> glycine responses (determined for each oocyte).

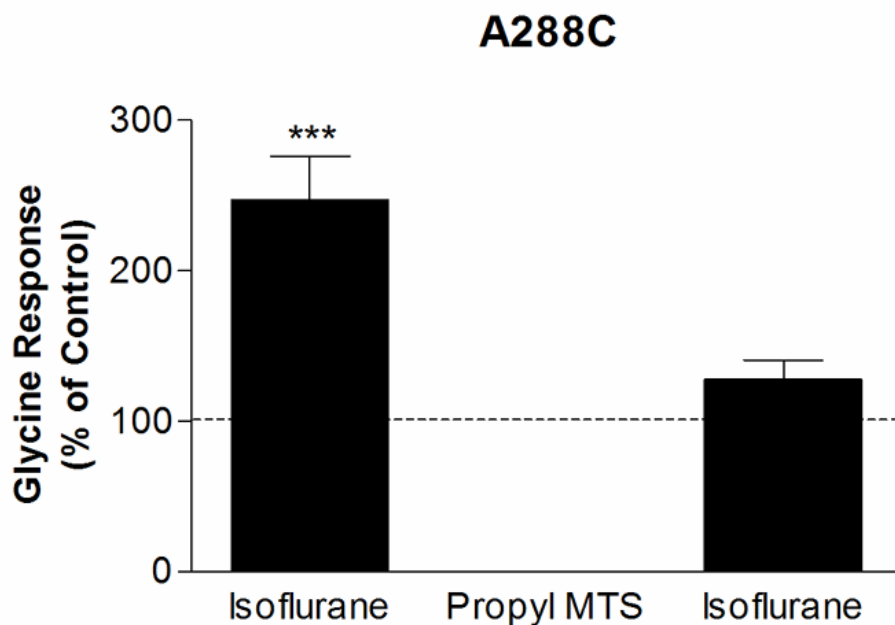
Glycine Receptor	Percent of Control		
	Ethanol	Octanol	Isoflurane
WT	180 $\pm$ 11	270 $\pm$ 17	550 $\pm$ 50
A288C	90 $\pm$ 13 ***	130 $\pm$ 23 ***	250 $\pm$ 30 ***

Data are expressed as a percent of control of the mean  $\pm$  S.E. of 4 to 16 oocytes. Mutant responses were compared to the WT using the unpaired Student's *t*-test (\*\*\*  $p < 0.001$ ).

### **6.2.6 Response of A288C to Isoflurane Before and After Reaction with Propyl MTS**

A question that follows from these data is to ask whether the binding of MTS reagents at A288C blocks further potentiation of the glycine receptor response by alcohols and anesthetics. This is good evidence that the MTS reagents are occupying the drug binding cavity and preventing a drug molecule from causing its effects on the receptor. Previously, this experimental strategy was used previously to test the importance of the TM2 position S267 (Mascia et al., 2000).

Because the A288C mutant was not sensitive to the effects of ethanol or octanol, but was potentiated by isoflurane (0.8 mM), the effect of isoflurane was tested before and after application of propyl MTS (500  $\mu$ M). Permanent occupation of the cysteine at A288C by propyl MTS eliminated the usual ability of isoflurane to potentiate the channel function (Figure 23).



**Figure 23. Reaction of propyl MTS with GlyR  $\alpha$ 1(A288C) prevents potentiation by isoflurane.** Isoflurane (0.8 mM) potentiation of the EC<sub>5-10</sub> glycine was measured both before and after application of 500  $\mu$ M propyl MTS. The initial potentiation by isoflurane was statistically different from the control. Following reaction with propyl MTS, isoflurane did not cause any further potentiation of the glycine response. Data are expressed as the mean  $\pm$  S.E. of 9 and 6 oocytes for each bar, respectively. The paired Student's *t*-test was used to determine significance of the isoflurane potentiation of the EC<sub>5-10</sub> glycine responses and the control EC<sub>5-10</sub> glycine responses (\*\**p* < 0.01).

The presented results were obtained by using an EC<sub>5-10</sub> concentration of glycine, determined for each individual oocyte. Previously, reaction of propyl MTS with S267C receptors was shown to result in a leftward shift in the glycine concentration response curve with no change in the maximum glycine response (Mascia et al., 2000). Consistent with this, we found that current induced by 1 mM glycine in the A288C mutant was not significantly changed by exposure to propyl MTS.

### 6.3 Discussion

In the glycine receptors, the TM3 mutation A288C shows state-dependence of water-accessibility. This position is only reactive in the presence of glycine, indicating that channel gating increases accessibility of MTS to this position or increases the reactivity of the introduced cysteine. Accessibility studies of the site homologous to A288 in TM3 in the GABA<sub>A</sub> receptor found that this position was also reactive in both the closed and open states with the neutral propyl and hexyl MTS (Jung et al., unpublished results, personal communication) and with the charged sulfhydryl-specific reagent pCMBS<sup>-</sup> (Williams and Akabas, 1999). The compound MTSEA did not react with this position of the GABA<sub>A</sub> receptor in either condition (Williams and Akabas, 1999). These results are compared in Table 14.

**Table 14**

The reactivity data for the glycine receptors position A288 (Lobo et al., 2004a) is compared with accessibility studies in TM3 of the GABA<sub>A</sub> receptor with the neutral propyl and hexyl MTS (Jung et al., unpublished results, personal communication), MTSEA and the charged sulfhydryl-specific reagent pCMBS<sup>-</sup> (Williams and Akabas, 1999).

**Table 14 - TM3 Summary**

Position GlyRa1			Position GABAA α1						
	Lobo et al., 2004 propyl MTS			Jung et al. (unpublished) propyl and hexyl MTS		Williams and Akabas, 1999&2002 pCMBS-		Williams and Akabas, 1999&2002 MTSEA	
		no gly	glycine	no GABA	GABA	no GABA	GABA	no GABA	GABA
M287	NR	↑	I290	NR	NR	↓	NR	NR	NR
A288			A291	↓	↓	NR	NR	NR	NR
V289			V292	NR	NR	NR	NR	NR	NR
C290			C293 (WT)	NR	NR	NR	↓	NR	NR
L291			Y294	propyl↓ hexyl↑	propyl↓ hexyl↑	NR	NR	NR	↓
L292			A295	NR	NR	↓	NR	NR	NR
F293			F296	NR	NR	↓	NR	NR	NR
V294			V297	NR	NR	↓	NR	NR	NR
F295			F298	NR	NR	NR	NR	NR	NR
S296			S299	NR	NR	NR	NR	NR	NR
A297			A300			NR	NR	NR	NR
L298			L301	NR	NR	NR	NR	NR	NR
L299			I302	NR	NR	NR	NR	NR	NR
E300			E303	NR	NR	NR	NR	NR	NR
Y301-N305			F304-V307	NR	NR	NR	NR	NR	NR

NR = no reaction

↑ increased response

↓ decreased response

In contrast to the GABA<sub>A</sub> receptor accessibility results, our results for the glycine receptor showed distinct reactivity only in the presence of agonist. This may reflect a difference in the arrangement of the residues involved with alcohol/anesthetic between these two overall very similar inhibitory receptors. One piece of experimental evidence that may reflect this difference between these receptors is the finding that nonhalogenated, alkane anesthetics potentiate glycine receptors, but have little to no effect on GABA<sub>A</sub> receptors (Hara et al., 2002; Raines et al., 2001).

Previously, A288C was not observed to react with propyl MTS because of the lower concentration used (Mascia et al., 2000), suggesting that propyl MTS has less access to the position and a slower rate of reaction than what was measured for S267C. In comparing the reaction rate constant of propyl MTS in the presence of glycine at A288C with that of S267C, the reaction at the TM3 position is much slower. Since agonist is necessary for MTS reaction with A288C, but is not required for S267C, this could account for a slower reaction rate at the A288 position. The A288 position may have fewer water-accessible, open channel substates during which reaction can occur, making reaction a rarer event.

This measured difference in rate constants is consistent with studies of reaction rate at homologous positions in the  $\beta 2$  subunit of GABA<sub>A</sub> receptor (Bali and Akabas, 2004). This study compared the rates of pCMBS<sup>-</sup> with the corresponding TM2 and TM3 cysteine mutants. The authors reasoned that this difference in rates may be caused by electrostatic interaction between the negatively charged pCMBS<sup>-</sup> and the positively charged arginine at the 19' position in TM2 (one helical turn above the cysteine mutant in



TM2) (Bali and Akabas, 2004). They hypothesized that this could cause an increased dwell time on the TM2 face, a higher local concentration of pCMBS<sup>-</sup> and therefore a higher reaction rate for the TM2 position (Bali and Akabas, 2004).

In our studies, the neutral propyl MTS was used for chemical modification, making an explanation involving electrostatic interactions insufficient to explain our data. While the dwell time of MTS molecules at the TM2 face and S267 may be higher, another possible reason for this may be that the local environment surrounding A288 is not as favorable for reaction in comparison. In the glycine receptor, on the TM2 helical face S267C is neighbored by S270 (above) and M263 (below). Meanwhile, the residues neighboring A288 on the TM3 helical face are I285 (above) and L291 or L292 (below). While the serines on the TM2 face make this region polar, the alanine, isoleucine and leucine(s) on the TM3 face are in contrast very nonpolar. Since the MTS molecule has a permanent dipole, dipole-dipole interactions may attract MTS to the TM2 face predominantly, resulting in a greater reaction.

Upon permanent occupation of the cysteine at A288C by propyl MTS, the usual ability of isoflurane to potentiate the channel function was lost. Results for the glycine receptor A288C mutant are consistent with the proposal that inhaled anesthetics and *n*-alcohols are binding to a single location on the glycine receptor. First, this substitution eliminates the usual receptor response to ethanol and octanol. Second, the response of the receptor to isoflurane is abolished following irreversible binding by propyl MTS. Added to the previous mutagenesis studies (Mihic et al., 1997; Ueno et al., 1999; Wick et al., 1998; Yamakura et al., 1999), which indicated that A288 (and the equivalent position

in the GABA<sub>A</sub> receptor - A291) were critical for drug action on the receptor, these data are strong support that A288C is contributing to a binding site for alcohols and anesthetics.

## 7.0 Transmembrane Segment 4

### 7.1 Introduction to Transmembrane Segment 4

Among the four TM segments, TM4 is the least conserved and the most hydrophobic in terms of sequence. In the recently published 4Å nicotinic acetylcholine receptor structure, the TM4 segment was imaged to be a helix though it was noted to be less precisely positioned than the others (Miyazawa et al., 2003). TM4 and the other three transmembrane segments form a left handed bundle, and TM4 comes apart from this bundle toward the extracellular end (Miyazawa et al., 2003). Data indicates that TM4 is mostly lipid-facing, with probable contact points with transmembrane segments 1 and 3.

Experimental data also supports an alpha helical secondary structure for TM4. Tryptophan substitution mutants in the TM4 segment of the *Torpedo* acetylcholine receptor  $\alpha$  subunit showed that mutants with reduced activity all clustered along a single face of the helix away from the membrane lipids (Tamamizu et al., 2000). The periodicity of labeling by the hydrophobic, photoactivable probes 2-[<sup>3</sup>H]-diazofluorene and [<sup>125</sup>I]TID of the  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  subunits of the *Torpedo* acetylcholine receptor was also interpreted as being due to an alpha helical structure with a broad face of the helix in contact with lipids (Blanton and Cohen, 1992; Blanton and Cohen, 1994; Blanton et al., 1998a).

The amino acids involved with alcohol and anesthetic action are predicted to face the center of the transmembrane domain of each glycine receptor subunit. The putative binding cavity is at the center of the four transmembrane helices, with known amino acids from TM1, TM2 and TM3 lining the cavity. This makes it possible that amino acids from TM4 also contribute to this drug binding cavity. In the family of ligand-gated ion channels, there is evidence that TM4 residues may also play a role in alcohol and anesthetic binding and/or action. Presently, it is unknown which specific amino acids are contributing to the drug binding pocket. A study by Jenkins et al., twelve positions in the extracellular portion of TM4 were mutated to tryptophan in the  $\alpha 1$  subunit of the GABA<sub>A</sub> receptor (Jenkins et al., 2002). This study examined the effect of substituting a bulky amino acid in place of the wild type residues to change drug modulation by volatile anesthetics. A number of positions were found to either increase or decrease the effects of isoflurane, halothane and chloroform (Jenkins et al., 2002), and these data will be compared to our studies of TM4 in the glycine receptor. Additionally, the EC<sub>50</sub> GABA values of the mutant receptors were plotted vs. the residue positions and were fitted to a sine curve with a period of  $3.6 \pm 0.1$  residues (Jenkins et al., 2002). This lends further support to the alpha helical arrangement of TM4.

Experimentally, no one has determined accessibility for TM4 in any member of the ligand-gated ion channel family, so we were interested in testing whether TM4 had positions which were water-accessible. This would give us more information about the structure of TM4 and indicate possible candidate positions critical for drug action.

Twelve single cysteine mutants in TM4 were made using site-directed mutagenesis and sequenced. We chose the homologous positions to those in the GABA<sub>A</sub> receptor studied by Jenkins et al (2002). They were first tested for their responses to glycine and then tested for their accessibility to the propyl methanethiosulfonate. The ability of propyl MTS compound to irreversibly occupy these sites was tested under conditions where the channel is either closed or open using two-electrode voltage clamping in *Xenopus* oocytes.

Mutants demonstrating accessibility were tested to see if they were responsive to alcohols (ethanol and octanol) and anesthetics (isoflurane and chloroform) both before and after reaction with propyl MTS. This provided information on whether the position was affected by introduction of the cysteine itself, as well as indicating whether reaction with MTS could occlude further potentiation alcohol and anesthetic molecules.

## **7.2 Results**

### **7.2.1 Concentration Response Curve Data for the TM4 Mutants**

First, the glycine concentration response curves for each of the twelve mutants TM4 mutants were determined. The maximal glycine response was elicited at 1 mM glycine for all mutants except Y410C, which required 5 mM glycine for the maximal response. For each mutant, the data was fitted for each oocyte with nonlinear regression curve fitting and the EC<sub>50</sub> glycine and Hill coefficients were averaged. These values, as well as the average maximal response to glycine, were compared to the WT receptors. The EC<sub>50</sub> values were not significantly different from the WT by one-way ANOVA. As a trend; however, all of the mutants had a lower sensitivity to glycine with W407C and Y410C having the lowest sensitivities and largest right shifts of their concentration response curves. The Hill coefficients of the mutants also did not show significant changes from the WT receptors. The maximal currents from the Y406C and Y410C receptors significantly decreased (Table 15).

**Table 15**

Glycine EC<sub>50</sub>. Hill coefficients and maximal glycine currents for the wild type (WT) receptor and the cysteine substitution mutants studied in transmembrane segment 4. The glycine EC<sub>50</sub> and Hill coefficients were experimentally calculated from averaged concentration response curves of single oocytes. Data are expressed as a mean  $\pm$  S.E. of 4 to 10 oocytes.

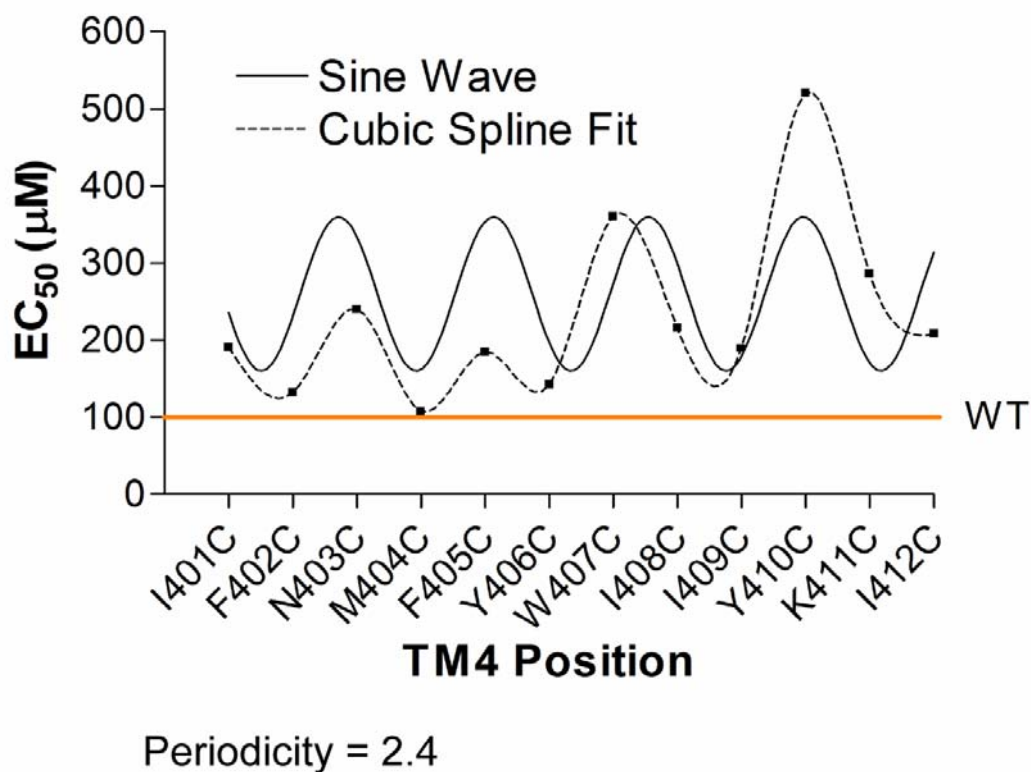
Receptor	EC <sub>50</sub> ( $\mu$ M)	Hill Coefficient	Max ( $\mu$ A)
WT	104 $\pm$ 5.8	3.2 $\pm$ 0.8	16.9 $\pm$ 1.2
I401C	198 $\pm$ 24	2.3 $\pm$ 0.2	11.9 $\pm$ 1.2
F402C	147 $\pm$ 24	3.5 $\pm$ 1.2	13.4 $\pm$ 1.9
N403C	283 $\pm$ 75	2.9 $\pm$ 0.2	15.1 $\pm$ 1.7
M404C	112 $\pm$ 11	2.1 $\pm$ 0.2	16.6 $\pm$ 1.4
F405C	190 $\pm$ 16	2.2 $\pm$ 0.2	21.3 $\pm$ 2.0
Y406C	162 $\pm$ 22	2.9 $\pm$ 0.6	7.4 $\pm$ 1.4**
W407C	731 $\pm$ 400	1.5 $\pm$ 0.2	13.8 $\pm$ 1.3
I408C	386 $\pm$ 180	2.6 $\pm$ 0.7	14 $\pm$ 1.6
I409C	197 $\pm$ 47	3.5 $\pm$ 0.5	13.6 $\pm$ 1.1
Y410C	553 $\pm$ 110	2.3 $\pm$ 0.2	8.2 $\pm$ 1.3**
K411C	290 $\pm$ 37	2.7 $\pm$ 0.4	13.2 $\pm$ 1.5
I412C	206 $\pm$ 15	2.5 $\pm$ 0.5	13.9 $\pm$ 1.1

\*\*  $p < 0.01$ ; significantly different from wild type receptors by one-way ANOVA with the Dunnett's post test.

### **7.2.2 Periodicity of TM4.**

By plotting the mean EC<sub>50</sub> values of the TM4 cysteine mutants versus their position, periodicity of the data was apparent. Using the cubic spline curve, the trend of the data points was shown. Using this curve as a reference, a sine wave curve was fit to the data and the periodicity was calculated to be approximately 2.4 amino acids per turn of the helix (Figure 24).



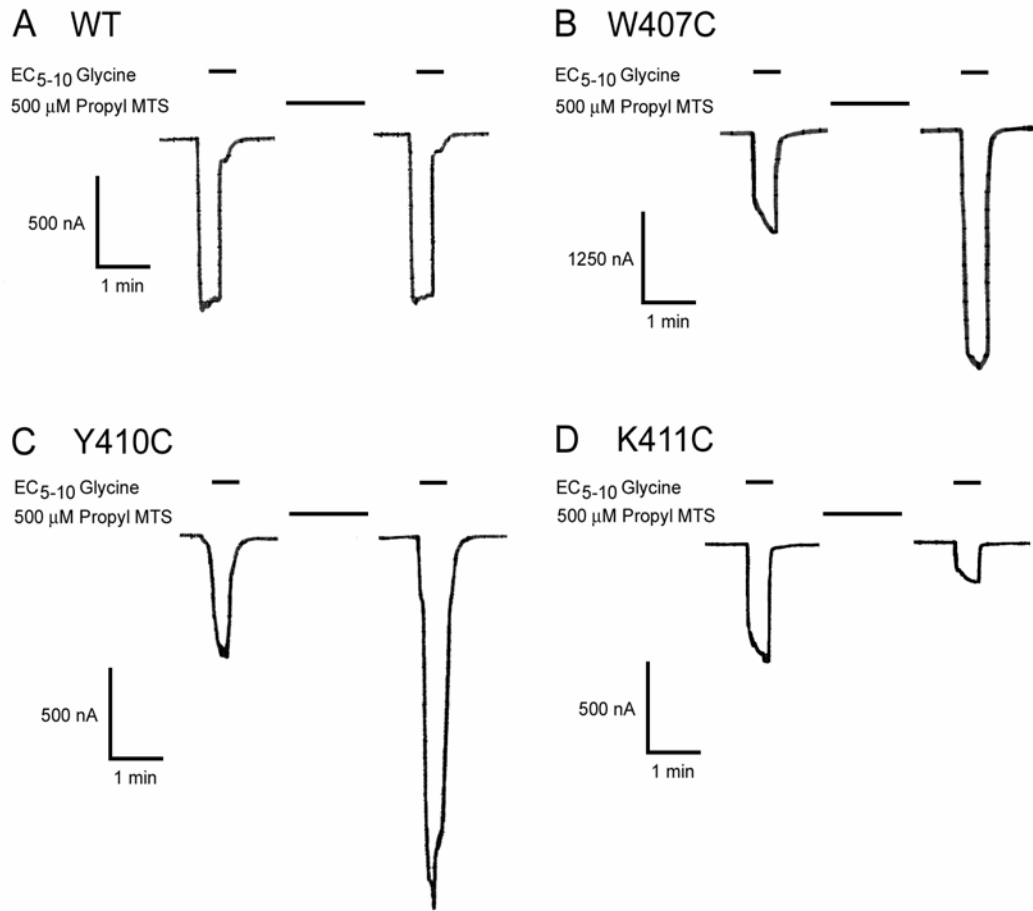


**Figure 24. The mean  $EC_{50}$  values of the TM4 cysteine mutants were plotted versus their position.** The mean  $EC_{50}$  glycine value of the WT is marked as a solid, straight line. All of the mutants had a greater  $EC_{50}$  value than the WT, with peaks at W407C and Y410C. The cubic spline curve shows the trend of the data points (dotted line). A sine wave curve (solid line) was fit to the data with the cubic spline as a reference. From the sine wave formula, the periodicity was calculated to be approximately 2.4 amino acids per turn of the helix.

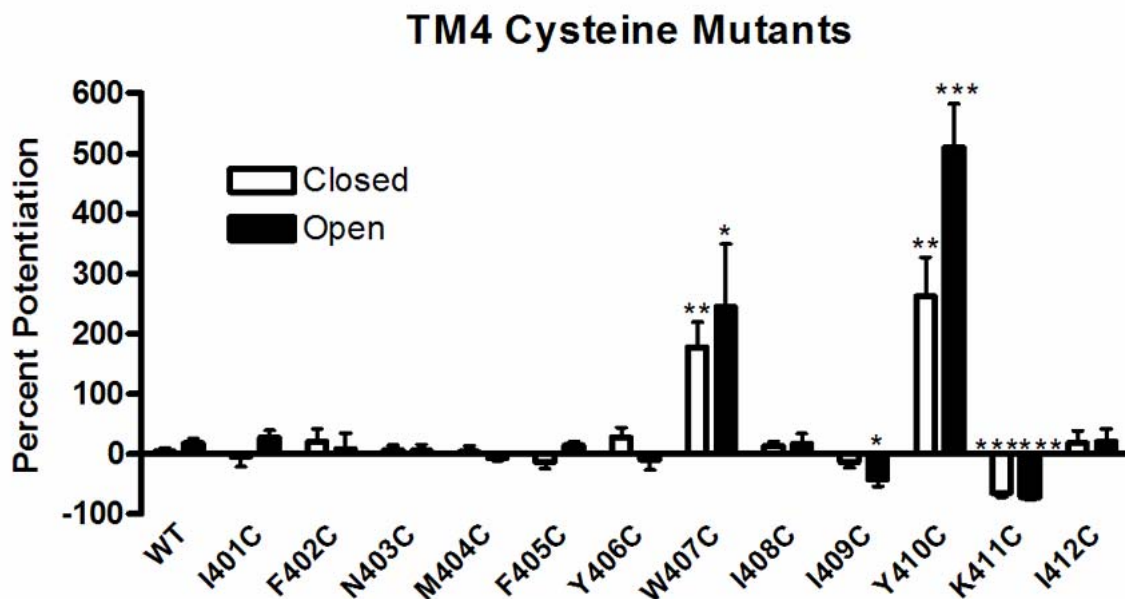
### 7.2.3 MTS Reactivity Summary

The twelve cysteine mutants and WT glycine receptor were tested for irreversible changes in receptor function after application of propyl MTS. The glycine EC<sub>5-10</sub> was applied 10 minutes before and 10, 20 and 30 minutes after application of 500  $\mu$ M propyl MTS. The initial and the EC<sub>5-10</sub> glycine responses at the 20 minutes time point was analyzed and compared. Accessibility was tested in both the presence and absence of a maximal glycine concentration. The maximal glycine concentration used for co-application with propyl MTS was 1 mM for the WT all of the mutants except for Y410C, where 5 mM glycine was used due to its lower sensitivity.

The WT and the intracellular six amino mutated positions (I401C to W406C) were inaccessible to reaction with propyl MTS in both the presence and absence of glycine. W407C reacted with propyl MTS, resulting in irreversible potentiation, under both conditions. I408C was inaccessible under both conditions. I409C only reacted in the presence of glycine, resulting in irreversible inhibition. Y410C reacted with propyl MTS in both the presence and absence of glycine, resulting in irreversible potentiation. K411C reacted in both conditions, resulting in irreversible inhibition of the glycine current. The final mutant, I412C, was inaccessible under both conditions. Representative tracings of the glycine responses before and after application of propyl MTS are shown for the WT, W407C, Y410C and K411C glycine receptors in Figure 25 and the mean reactivity data for all of the mutants and the WT receptor are shown in Figure 26.



**Figure 25. Effect of propyl MTS on WT, W407C, Y410C and K411C glycine receptors.** The glycine current resulting from an EC<sub>5-10</sub> of glycine was tested before and 20 minutes after application of 500  $\mu$ M propyl MTS. Experiments were conducted in the absence and presence of glycine (1 mM). Representative tracings are shown for the WT and three reactive mutants for closed state experiments. A) The glycine current of the WT was not altered following application of propyl MTS. B) and C) Both W407C and Y410C were irreversibly enhanced after application of propyl MTS in the absence of glycine. D) K411C showed irreversible inhibition following reaction of propyl MTS.



**Figure 26. Accessibility of propyl MTS to GlyR  $\alpha 1$  transmembrane 4 mutants.**

Propyl MTS (500  $\mu$ M) was applied to receptors in the absence (Closed) and presence (Open) of a maximal concentration of glycine. Four mutants reacted with propyl MTS to produce a change in the glycine  $EC_{5-10}$  response. W407C and Y410C reacted in both the presence and absence of glycine and had a potentiated response to glycine. I409C and K411C reacted with propyl MTS and showed inhibition of the initial glycine response. I409C only reacted with propyl MTS in the presence of glycine, while K411C reacted in both states. Data are expressed as a mean  $\pm$  S.E. of 5 to 11 oocytes. All mutant responses were compared to the respective WT control by one-way ANOVA and the Dunnett's post test (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ).

#### **7.2.4 WT, Y410C and K411C Responses to Alcohols and Volatile Anesthetics**

The responses of two of these accessible mutants in TM4 to alcohols and anesthetic were determined and compared to the WT receptor. Percent of control effects of ethanol (100 mM), octanol (115  $\mu$ M), isoflurane (0.7 mM), and chloroform (2.0 mM) were tested on an EC<sub>5-10</sub> concentration of glycine, which was determined for each oocyte. The Y410C mutant showed no potentiation to ethanol and significantly reduced potentiation to octanol. K411C also showed no potentiation to ethanol, and additionally had a reduced isoflurane effect (Table 16).

**Table 16**

Percent of control responses of WT, Y410C and K411C receptors to ethanol (100 mM), octanol (115  $\mu$ M), isoflurane (0.7 mM) and chloroform (2.0 mM). Percent of control responses were measured on the EC<sub>5-10</sub> glycine responses (determined for each oocyte).

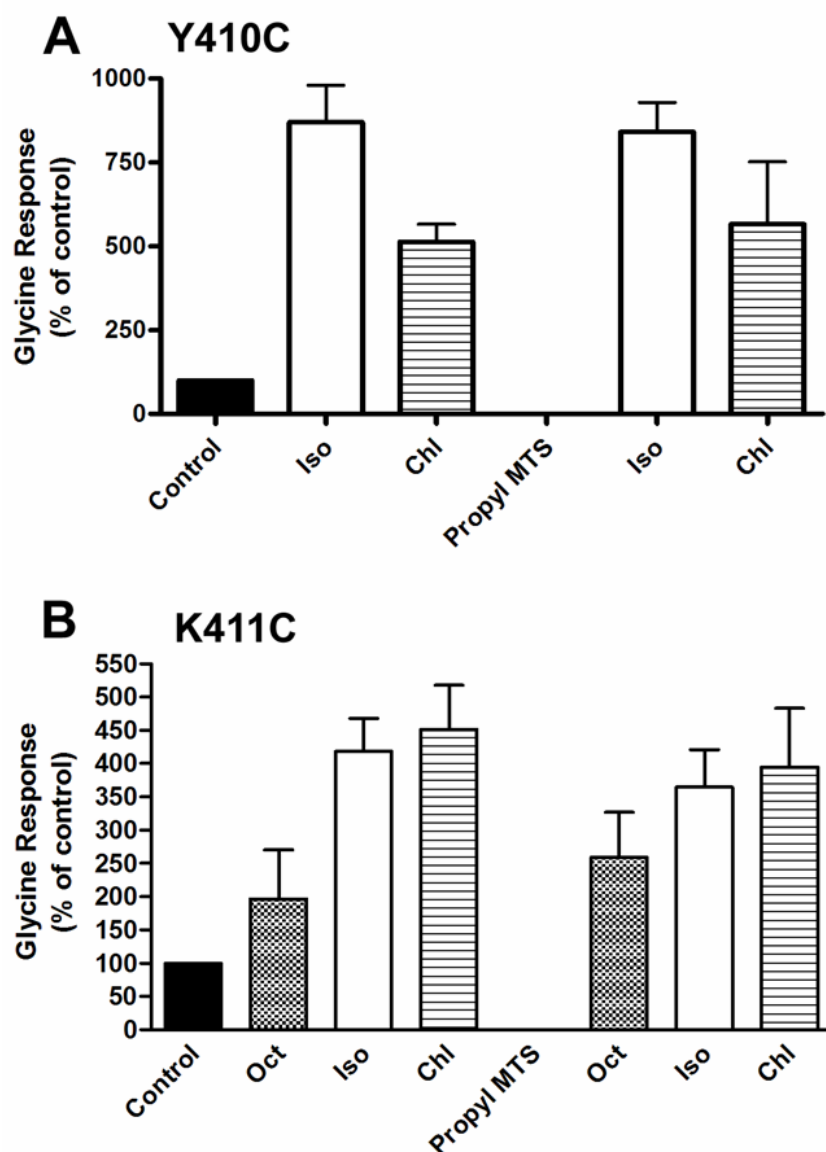
Glycine Receptor	Percent of Control			
	Ethanol	Octanol	Isoflurane	Chloroform
WT	180 $\pm$ 11	270 $\pm$ 17	820 $\pm$ 82	300 $\pm$ 35
Y410C	62 $\pm$ 10 **	140 $\pm$ 20 *	870 $\pm$ 110	510 $\pm$ 52
K411C	85 $\pm$ 5 **	200 $\pm$ 74	420 $\pm$ 49 **	450 $\pm$ 66

Data are expressed as a percent of control of the mean  $\pm$  S.E. of 4 to 18 oocytes. Mutant responses were compared to the WT using one-way ANOVA and Dunnett's post-test (\*\*  $p < 0.01$ ).

### **7.2.5 Effects of Octanol, Isoflurane and Chloroform on Y410C and K411C Before and After Reaction with Propyl MTS**

Since both Y410C and K411C react with MTS and are water-accessible, we asked if the binding of MTS reagents at these positions could block further potentiation of the glycine receptor response by alcohols and anesthetics as shown previously (Mascia et al., 2000). If MTS reaction blocked further drug effects at these receptors, this would indicate that the MTS reagents were permanently occupying the drug binding cavity and thereby preventing another drug molecule from binding to and effecting the receptor.

The effects of isoflurane (0.7 mM) and chloroform (2.0 mM) were tested on the EC<sub>5-10</sub> glycine response of the Y410C. Since K411C responded to octanol (115  $\mu$ M), this alcohol was tested in addition to the two anesthetics on this receptor. Next, propyl MTS was applied in the absence of glycine (90s) for reaction with the receptors, and the maximal glycine response and EC<sub>5-10</sub> glycine concentration was re-determined. Then the ability of propyl MTS (500  $\mu$ M) to alter the responses of these two volatile anesthetics (and octanol for K411C) was determined. Application of propyl MTS did not reduce or eliminate the effects of these drugs on the potentiation of the Y410C and K411C mutants (Figure 27).



**Figure 27. Effects of alcohols and anesthetics on Y410C and K411C receptors before and after application of propyl MTS.** The potentiation of the glycine  $EC_{5-10}$  by isoflurane (Iso; 0.7 mM) and chloroform (Chl; 2.0 mM) were measured before and after the application of 500  $\mu$ M propyl MTS (in MBS). Octanol (Oct; 115  $\mu$ M) was tested only on K411C because it did not significantly potentiate Y410C receptors. A) Application of propyl MTS to the Y410C receptor produced no change in the response of the receptor to isoflurane and chloroform. B) Application of propyl MTS to the K411C receptor produced no change in the responses of the receptor to octanol, isoflurane and chloroform. The Student's paired  $t$ -test was used to determine differences in the mean drug potentiation from before and after the propyl MTS application ( $p > 0.05$  for all comparisons).



### 7.3 Discussion

After mutagenesis of twelve positions in TM4 to cysteine and reaction with MTS reagents, water-accessible in the transmembrane 4 segment were determined. Four positions in the extracellular portion of TM4 react with to produce an irreversible change in glycine receptor function. This is the first accessibility study on TM4 in any ligand-gated ion channel.

The period of the EC<sub>50</sub> values the cysteine mutants plotted vs. position for was approximately 2.4 residues. This is less than the period of  $3.6 \pm 0.1$  residues estimated by from the EC<sub>50</sub> GABA values of TM4 tryptophan mutants (Jenkins et al., 2002). One reason for this may be that the alpha helical arrangement of TM4 may be disturbed to a greater extent with cysteine mutations than with tryptophan mutations.

The four reactive positions; W407, I409, Y410 and K411 were all located in the most extracellular portion of TM4. Because TM4 is hypothesized to be surrounded by lipids, this is a somewhat surprising result. The reactivity data indicates that these positions are in a water-filled environment. Both W407C and Y410C were reactive in both the absence and presence of glycine. Reaction at these positions resulted in an increased response to glycine. I409C was reactive only in the presence of glycine and resulted in a decreased current. The last reactive mutant, K411C, was reactive in both the absence and presence of glycine, and reaction resulted in an irreversible decrease in receptor current.

One aspect that complicates analysis of these data is the relative size of the native amino acids in TM4. Overall, in the glycine receptor, the 12 amino acids targeted for

mutation were large. Meanwhile, cysteine is a small amino acid in comparison. With each introduced cysteine, the mutation itself may introduce a water-filled cavity where MTS may react. These substitutions may enlarge existing water cavities or introduce new pockets of water. This means it is possible that the native amino acid is not in a water-filled cavity in the wild type receptor. Examples of this might include W407 and I409, two very hydrophobic amino acids, which are unlikely to be in a water-filled environment. However, there is no alternative method to alleviate this problem, since cysteine is the only amino acid we can target with thiol-reactive reagents. Until a high resolution crystal structure of TM4 is available for the glycine receptor, the precise orientation and surrounding environment of these residues will not be without question.

Both the W407C and Y410C mutants have increased  $EC_{50}$  values and are less sensitive to glycine in comparison to the WT receptor. Also, both of these mutants showed potentiation of the glycine response after reaction with propyl MTS. One possible explanation of this is that the binding of propyl MTS at each of these introduced cysteines increases the volume occupied at these position to mimic the native amino acids. Thus, reaction of propyl MTS could cause the mutant receptors to mimic the WT receptor to result in a leftward shift the glycine concentration response curve. Another notable point is that these two amino acids should lie along the same helical face if TM4 is an alpha helix. This face of TM4 could be interacting with another TM region or part of the protein to regulate channel gating, and possibly drug action in the same manner.

At present, two of the four accessible positions were tested to see if reaction with propyl MTS could block further potentiation by octanol and volatile anesthetics. In

contrast to S267C (Mascia et al., 2000) and A288C (Chapter 6), propyl MTS reaction did not block further potentiation by drugs at Y410C and K411C. Y410C was potentiated by isoflurane and chloroform both before and after reaction with propyl MTS. The potentiation of K411C by octanol, isoflurane and chloroform was also unchanged by covalent reaction with propyl MTS. One possible reason is that the introduced MTS reagent is not large enough or not the correct shape to mimic a drug molecule in the cavity, and therefore prevent drug action. Reaction with a larger MTS reagent may be necessary to fill more of the drug binding pocket.

Alternative explanations may be that these two positions are not in the drug binding pocket, or that they are located in the alcohol and volatile anesthetic binding cavity but are not playing a direct role in binding these drugs. These cysteine mutations themselves did affect drug action. Y410C did not respond to ethanol or octanol, while K411C was not potentiated by ethanol and had a reduced isoflurane effect. Although, propyl MTS did not prevent alcohol/anesthetic potentiation, it is not yet possible to exclude these two residues as candidates for participation in a drug binding cavity.

To this date, there have been no other SCAM studies on TM4 on any other ligand-gated ion channels. For this reason, these data were compared to published data on tryptophan mutants in TM4 of the *Torpedo californica* nicotinic acetylcholine receptor  $\alpha$  subunit (Tamamizu et al., 2000) and the  $\alpha 1$  subunit of the GABA<sub>A</sub> receptor (Jenkins et al., 2002) (Table 17). These sequences were aligned using the consensus sequences of the ligand-gated ion channels (Bertaccini and Trudell, 2002). The amino acids postulated to be lipid-facing in the acetylcholine receptor were not water-accessible in the glycine

receptor with the exception of I409C. I409C showed a decrease in receptor function after reaction in the presence of glycine, indicating a possible shift from a lipid-facing to a water-facing position. Another possibility is that the mutation from isoleucine to cysteine created a new water pocket in a previously unexposed position. Also, the glycine receptor mutant W407C did not cause any significant change in receptor, while mutation at the aligned position in the acetylcholine receptor (V423W) resulted in decreased expression. Comparison of our glycine receptor results with those of the GABA<sub>A</sub> receptor showed some overlap, and also differences. Jenkins et al. tested whether tryptophan mutations altered receptor sensitivity to three volatile anesthetics: isoflurane, halothane and chloroform (Jenkins et al., 2002). While there was no reactivity with MTS observed for positions I401, F402, Y406 and I412, mutation to tryptophan could alter anesthetic effects. The glycine receptor mutant W407C was reactive, but could not be compared to the GABA<sub>A</sub> receptor results, where the position was already a tryptophan. Meanwhile, overlap between these data sets occurred with the three other water-accessible positions. Positions I409, Y410 and K411 were all water accessible in the glycine receptor, and caused changes in anesthetic action when mutated to tryptophan in the GABA<sub>A</sub> receptor (Table 17).

**Table 17**

Since no other SCAM studies have been completed on TM4, the glycine receptor TM4 reactivity data is compared with published data on tryptophan mutants in the TM4 segments of the *Torpedo californica* nicotinic acetylcholine receptor  $\alpha$  subunit (Tamamizu et al., 2000) and the  $\alpha 1$  subunit of the GABA<sub>A</sub> receptor (Jenkins et al., 2002).



Although it is not certain at this point if the four water-accessible TM4 mutations are contributing to an alcohol and anesthetic binding cavity, these positions have been defined as candidates worthy of further study.

## 8.0 Conclusions and Discussion

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Lobo IA, Trudell JR, Harris RA. Cross-linking of glycine receptor transmembrane segments two and three alters coupling of ligand binding with channel opening.

*J Neurochem.* 2004 Aug;90(4):962-9.

Lobo IA, Mascia MP, Trudell JR, Harris RA. Channel gating of the glycine receptor changes accessibility to residues implicated in receptor potentiation by alcohols and anesthetics. *J Biol Chem.* 2004 Aug 6;279(32):33919-27. Epub 2004 May 28.

This thesis work has further defined the putative alcohol and volatile anesthetic binding site in the glycine receptor. The use of mutagenesis, crosslinking, and probing with sulfhydryl-specific reagents has identified new characteristics of this cavity. The interface between TM2 and TM3 has been located and better defined by crosslinking two



amino acids known to be critical for volatile anesthetic and alcohol action. Water-accessible positions were identified in all four transmembrane segments by reaction with sulfhydryl-specific probes. Additionally, conformational changes, as demonstrated by changes in reactivity to MTS reagents, were observed for positions in all four transmembrane segments with channel gating.

### **Positioning Transmembrane Segments**

The results presented in Chapter 3 suggest that an intrasubunit disulfide bond forms between S267C and A288C in GlyR  $\alpha$ 1(S267C/A288C) receptors. These two amino acids have been shown to be critical for alcohol and volatile anesthetic action and hypothesized to line a binding pocket for alcohols and volatile anesthetics between the four transmembrane segments in the GlyR subunit (Jenkins et al., 2001; Mascia et al., 2000; Mihic et al., 1997; Wick et al., 1998; Yamakura et al., 2001). By crosslinking these two residues, the close positioning of these two amino acids was determined.

Our results indicate that S267 and A288 face a common pocket within each subunit. Also, crosslinking allowed the distance between the alpha carbons of S267 and A288 to be estimated at approximately 7 Å. Since the vertical position of TM2 with respect to TM3 was previously undetermined, disulfide bonding between these two introduced cysteines provides insight regarding the location and role of the TM2-TM3 interface.

With respect to these two positions and the newly identified water-accessible positions in TM2, TM4 and TM1, it would be useful to create double mutants to see if

crosslinking occurs between other pairs of transmembrane segments. Many of the cysteine mutants tested resulted in a reduction or a loss of drug potentiation. Of these positions, it is not yet certain whether these amino acids also line the drug binding cavity. If they do participate directly in drug binding, then knowledge of the orientation of these amino acid side chains would be very useful. Not only would this provide information on the alcohol and anesthetic binding cavity, crosslinking (via disulfide reaction, or crosslinking reagents) would provide structural data to position transmembrane segments more definitively.

### **How Does Channel Gating Alter Glycine Receptor Conformation?**

The gating mechanism of ligand-gated ion channels is not entirely understood. The binding of small neurotransmitters at locations in the extracellular domain, far from the pore, causes conformational changes in the transmembrane region, allowing for ion conductance. The gating mechanism of ion channels may involve rearrangement of a sub-region within the pore itself, the entire pore, or may cause the channel structure to be even more substantially altered. Because the energy of agonist binding is small, it would be difficult to explain any major structural movement in the protein during gating. Also, it would be unfavorable for the rearrangements to be extensive since the channel must soon close. Better supported experimentally is a cascade model involving local changes that rearrange low energy interactions (Kash et al., 2004). From ligand binding, the signal is transduced through the N-terminal extracellular domain, through the TM2-TM3 linker (Kash et al., 2003) and causes a twisting of the barrel of TM2 helices to open the

channel (Unwin, 1995; Unwin, 1998). The opening of the channel has been modeled by equivalent 15 degree rotations of all five TM2 helices (Unwin, 2003).

When the transduction signal reaches the transmembrane domain, TM2 moves. It would make sense that TM3 may move as well, causing the state-dependent change in accessibility observed at A288. While it is obvious that the lining of the channel changes conformation with channel gating and that TM3 may move because of transduction through the linker, it is perhaps unexpected that TM1 and TM4 also show differences in accessibility with channel gating. Previous studies have identified differences in accessibility in receptor subunits when neurotransmitter was present or absent. From these data, amino acids in all four transmembrane segments of the glycine receptor have alterations in accessibility with channel gating. Meanwhile, other positions were always in water-accessible and reactive positions, or were inaccessible under all conditions tested.

### **Conformational Changes in the Alcohol and Anesthetic Binding Cavity**

Our results on the TM2 mutant S267C indicated that conformational changes occurring during channel gating increased the size of the binding cavity. While short chain MTS reagents reacted in the closed state of the receptor, both short and longer chain MTS compounds reacted in the presence of glycine. Additionally, our data showed increased accessibility or reactivity in the open state of the channel, indicating changes in the local environment of area of the protein.

For both I229C and A288C, there is a distinct conformational change occurring with channel gating that allows MTS to react only in the presence of glycine. The state-dependence of reaction indicates that there are specific conformational changes with channel gating occurring even in TM1 and TM3 of the GlyR, along with the necessary changes in TM2, completely altering the accessibility to these two positions. Previously, A288C was not observed to react with propyl MTS because of the lower concentration used (Mascia et al., 2000). A slower reaction rate with propyl MTS was measured for A288C than the rate of reaction at S267C. This is most likely because propyl MTS has less access to the A288 position or the local environment is not as conducive to reaction as that surrounding S267. Another TM2 position, M263C, reacted with propyl MTS, but not with decyl MTS, demonstrating that compounds of this length are too large to access this cysteine. For S270C, propyl MTS reacted in both states, but the larger decyl MTS reacted only in the presence of glycine, indicating that the water-filled space around this position increased in the open state.

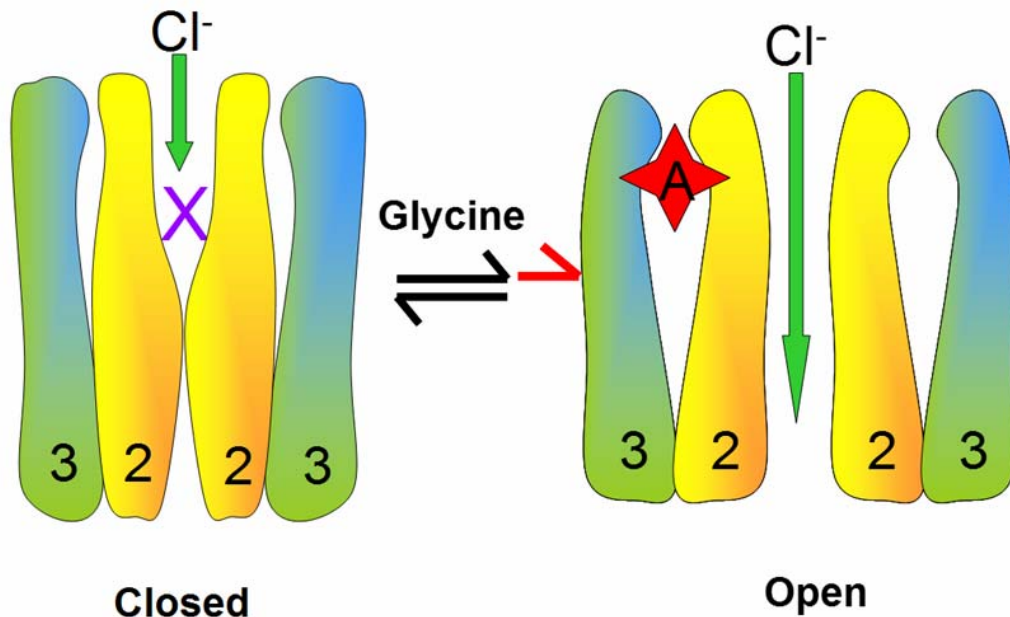
Though the positioning of the amino acids in TM4 are not yet definitive, four cysteine mutants were accessible to propyl MTS: W407C, I409C, Y410C and K411C. I409C was only accessible in the presence of glycine, while the other three amino acids were accessible in both the closed and open state. Because these four amino acids, located in the extracellular portion of TM4 where they could face the other transmembrane segments and are water-accessible, these positions are candidates for participating in the alcohol and anesthetic drug binding cavity. The same holds true for the TM1 position I229. Further experiments to test if longer chain MTS reagents can

block potentiation by drugs and crosslinking experiments would lend more evidence to determine whether these positions are playing a role in drug binding or not.

### **Proposed Mechanism for Drug Action**

Our results lead us to a potential mechanism for alcohol and anesthetic action on the glycine receptor and other related ligand-gated ion channels. Because we have evidence that the site of action of alcohols and volatile anesthetics experiences conformational changes during channel gating, this in turn suggests that drugs occupying this pocket may stabilize the open state of the channel to produce their effects (Figure 28).

## A Model of Alcohol/Anesthetic Action



**Figure 28. Schematic view showing a slice view through a glycine receptor transmembrane region.** Only two pairs of transmembrane 2 and 3 segments are shown for a single glycine receptor. When glycine is not present, the channel is closed, and no chloride ions can flow through the pore lined by the TM2 segments. Upon the addition of glycine, the channel gate can open allowing for conductance through the pore. We hypothesize that alcohols or volatile anesthetics (A) bind in a water-filled cavity formed in part by residues from TM2 and TM3, which results in stabilization of the channel's open state.

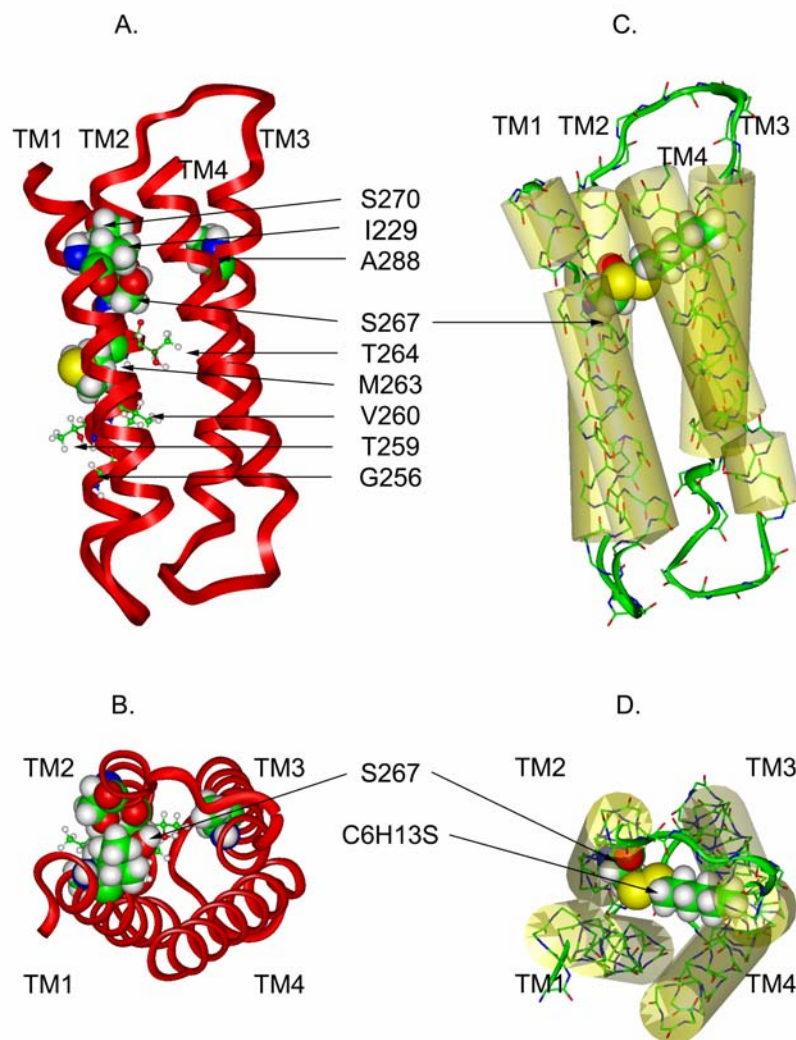
## **Molecular Modeling of the Alcohol and Volatile Anesthetic Binding Cavity**

The structural model of GlyR  $\alpha 1$  derived by Trudell and Bertaccini (in press) shows S267 and A288 in proximity (Figure 29). The proximity of these two residues and their orientation toward the center of the subunit is consistent with studies that showed additivity of the side chain volumes of these residues in changing the cutoff of long chain alcohols (Wick et al., 1998) and potentiation by anesthetics (Jenkins et al., 2001). In addition, the proximity of these residues is consistent with the ability of a double mutation (S267C/A288C) to form disulfide bonds (Lobo et al., 2004b). Although it is possible for side chains of distant residues to form disulfide bonds during thermal-motion induced excursions from their mean positions, the highest reaction rate is expected when the C $\alpha$  to C $\alpha$  distance is approximately 6 Å (Lobo et al., 2004b).

Experiments conducted on five mutants; I229C, M263C, S267C, S270C and A288C, provide evidence that accessibility to the region of the putative alcohol and anesthetic binding cavity changes with channel gating. Under different conditions, MTS compounds covalently reacted at these positions to result in enhancement of glycine receptor function. These data are shown in a homology model of GlyR  $\alpha 1$  (Trudell and Bertaccini, 2004; Yamakura et al., 2001), where the reactive positions are rendered with space-filling surfaces, while the non-reactive positions tested are shown as ball and stick surfaces (Figure 29A and 29B). The disulfide bond to hexyl sulfide (formed after the reaction with hexyl MTS) was modeled for the S267C receptor. Positioning was based by forming the disulfide bond and then re-optimizing the GlyR model with harmonic

restraints on all the backbone atoms of the subunit to illustrate a likely orientation and show the scale of the molecule relative to the subunit (Figure 29C and 29D).





**Figure 29. Molecular model of the transmembrane domain of one subunit of GlyR  $\alpha 1$  that was built by threading the primary sequence of GlyR  $\alpha 1$  onto a template of a four-helical bundle.** A) The homology model viewed from the side in the plane of the membrane shows a putative alcohol and anesthetic binding pocket, a cavity in the center of the receptor subunit. Residues that did react with MTS reagents are rendered with space-filling surfaces (I229, M263, S267, S270, and A288), and those that did not are rendered with ball and stick surfaces (G256, T259, V260, T264). The peptide backbone is shown as a red ribbon. B) The same model as A) viewed from the extracellular surface and looking into the center of the four-helical bundle. C) The model viewed from the side in the plane of the membrane with a disulfide bond to hexyl sulfide (formed after the reaction with hexyl MTS) from the sulfur atom of S267C in the mutated receptor. The four alpha helices are rendered as transparent yellow cylinders, the random coils as green ribbons. D) The same model as C viewed from the extracellular surface and looking into the center of the four-helical bundle.

## **Discussion of SCAM data**

As in other studies using sulfhydryl reagents as probes, our experimental design leaves the native cysteines of the receptor intact (Akabas et al., 1992; Karlin and Akabas, 1998; Mascia et al., 2000). Observing no change in the WT response following any of the MTS reagents used, we assume that mutant receptors have a structure and properties similar to the WT receptors and that enhancement is due to specific reaction of MTS at the introduced cysteines.

As with all mutagenesis studies, it is important to keep in mind the limitations that are inherent to trying to examine a WT proteins's function in an altered protein. The single amino acid substitutions are presumed to change only local conformations rather than cause substantial alterations in receptor structure. We must keep in mind that definitive placement of an alcohol and volatile anesthetic binding site will only be accomplished when a glycine receptor is crystallized with a bound alcohol or anesthetic molecule.

## **What is an Alcohol and Volatile Anesthetic Binding Site?**

We are left with the question of what constitutes an alcohol and volatile anesthetic binding site. Since the putative alcohol and volatile anesthetic binding cavity is composed of amino acids contributed from different receptor segments, rather than being composed of a single stretch of amino acids in more easily identifiable motif, it is a greater challenge to identify all of the positions contributing to these cavities. Also, it will be a challenge to compare the binding cavity in the glycine receptor to those in other

proteins known to be affected by alcohols and anesthetics. At present, it is not possible to search through high resolution, tertiary protein structures to identify drug binding sites in proteins known to respond to drugs, or to find candidate proteins which may bind alcohols and anesthetics.

We are still learning about what constitutes an alcohol and volatile anesthetic binding site in a few known proteins. From experiments using firefly luciferase, a soluble protein which is inhibited by anesthetics, it was suggested that anesthetics bind to an amphipathic cavity of defined dimensions (Franks and Lieb, 1985). Also, crystallographic data of the anesthetic bromoform bound to the firefly luciferase enzyme showed that there were minimal changes in overall protein structure as a result of anesthetic binding (Franks et al., 1998).

A recent study on the odorant binding protein LUSH from *Drosophila melanogaster* showed that short-chain alcohols bound to a single site (Kruse et al., 2003). This cavity is normally hydrated. When ethanol or butanol occupied the binding site between two alpha helical segments, the protein's conformation was stabilized profoundly. Water is described as an ideal ligand for displacement from drug binding cavities, serving to increase the binding energy of drug molecules (Trudell and Harris, 2004). Thus, as in LUSH, the binding of small drug molecules, like alcohols and volatile anesthetics, have the ability to stabilize protein conformations.

The theme of alcohols and anesthetics binding between alpha helices, or within pockets formed by turn/loop regions and their adjacent alpha helices was suggested in an analysis of protein segments characterized for alcohol/anesthetic binding by high-

resolution 3-D structures and mutagenesis (Dwyer and Bradley, 2000). It was concluded that alcohols mainly act as hydrogen bond donors with other hydrophobic interactions stabilizing binding (Dwyer and Bradley, 2000). In LUSH, a motif of amino acids containing hydroxyls formed the alcohol binding site (Kruse et al., 2003). Kruse et al. speculated that this motif of serines and threonines could be conserved in other proteins (Kruse et al., 2003). The role of one of these serines in the glycine receptor could be served by S267.

Our data for the glycine receptor suggest that alcohols and volatile anesthetics bind to a water-filled protein cavity that is formed by at least two alpha helical segments. While we are not yet at a stage to compare the final structure alcohol and anesthetic binding site of the glycine receptor with those in other proteins, the use mutagenesis, biochemistry and molecular modeling have advanced the available structural information. From the known data, the glycine receptor's drug binding pocket is amphipathic, with hydrophilic regions contributed by S267 in TM2 (and possibly TM4) function as a hydrogen bond acceptor. Meanwhile hydrophobic amino acids in TM3 (A288), along with TM1 and TM4, could serve to stabilize drug binding for the hydrophobic end of the drug molecule. In the glycine receptor, occupation of this site by alcohols and volatile anesthetics could act to stabilize the open conformation of the receptor.

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## Vita

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